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TITLE

SURVIVAL AND PHYSIOLOGICAL STATUS OF
ESCHERICHIA COLI IN LAKE WATER UNDER
DIFFERENT NUTRIENT CONDITIONS

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SURVIVAL AND PHYSIOLOGICAL STATUS OF
ESCHERICHIA COLI IN LAKE WATER UNDER
DIFFERENT NUTRIENT CONDITIONS

RESIT ÖZKANCA

A Thesis presented for the degree of
Doctor of Philosophy

Department of Biological Sciences,
University of Warwick

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Declaration

I declare that no material contained in this thesis has been used in any other submission for an academic award.

Resit ÖZKANCA

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SURVIVAL AND PHYSIOLOGICAL STATUS OF *ESCHERICHIA COLI* UNDER DIFFERENT NUTRIENT CONDITIONS IN LAKE WATER.

Survival of *Escherichia coli* has been examined under the effect of various environmental factors, nutrient and stress conditions in natural lake water or sterile microcosms. *E. coli* could survive for at least 200 days at temperatures below 25°C under starvation stress in sterile lake water. Whilst *E. coli* survived for the longest period of time at 4°C, the shortest survival time was always at 37°C in both natural and sterile lake water microcosms. Predation by protozoa was not found to be a significant factor affecting the survival of *E. coli* in natural lake water microcosms. The addition of sources of nutrients to lake water enhanced the survival of *E. coli* in both natural and sterile lake water. *E. coli* could grow in the lake water with the addition of a single carbon source or single amino acid addition but ultimately there was an accelerated decline in numbers. On the other hand the addition of ammonium sulphate and casein enhanced survival and allowed growth. The measurement of metabolic enzyme activities such as succinate and NADH dehydrogenase activity showed that *E. coli* reduced its metabolic activity very quickly upon starvation especially at 37°C. The reduction in activity was again temperature dependent. *E. coli* cells also reduced in size under starvation stress in lake water and the rate at the size reduced was temperature dependent. Measurements of the respiratory activity of the cells showed that although the viable count declined below detection limits under starvation stress, at least 50% of the cells could still respire. This indicates that the cells enter a viable but non-culturable phase in lake water. Alkaline phosphatase activity increased in cells which were incubated under starvation conditions. Pretreatment of the cells with alcohol, hydrogen peroxide or salt to induce stress proteins also lead to an increase in alkaline phosphatase activity. The addition of nutrient sources, particularly amino acids, to sterile lake water microcosms increased the activity of alkaline phosphatase and β -galactosidase enzymes. Two-dimensional gel electrophoresis was used to show changes in the protein patterns of *E. coli* affected by starvation and other stress conditions. Some proteins were induced under starvation conditions, others were repressed. The changes in the relative concentration of the outer membrane proteins, OmpC, OmpF and OmpA were quantified. All were affected by starvation stress with OmpA disappearing from the outer membrane. A 45 kDa protein was induced under all stress conditions. Other stresses resulted in the induction of a 70 kDa protein (oxidation stress by hydrogen peroxide) or the complete disappearance of the major outer membrane proteins (sodium dodecyl sulphate treatment). The survival of *E. coli* under starvation and other stress conditions was correlated with the induction of specific proteins.

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Abbreviations

μ	:	10^{-6} gram.
μm	:	10^{-6} meter.
2-D gel	:	Two dimensional gel electrophoresis.
AODC	:	Acridine orange direct count.
C	:	Carbon.
cfu	:	Colony forming units.
d	:	Day.
DCIP	:	2,6-dichlorophenolindophenol.
DNA	:	Deoxyribonucleic acid.
DNase	:	Deoxyribonuclease.
EDTA	:	Ethanediaminetetraacetic acid.
ETS	:	Electron transport system.
Fig.	:	Figure.
g	:	Gram.
g	:	Gravitational force.
g-C/L	:	Gram carbon in a litre.
g-N/L	:	Gram nitrogen in a litre.
h	:	Hour(s).
htpR	:	Heat shock regulatory protein.
INT	:	2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride
kDa	:	kilodalton.
L	:	Litre.
M	:	Molarity.
mA	:	Milliamper.
mg	:	Milligram.

mg/L	:	Milligram per litre.
mg/ml	:	Milligram per millilitre.
MIC	:	Minimum inhibitory concentration.
min.	:	Minutes.
MPN	:	Most probable number.
M_r	:	Relative molecular mass.
N	:	Nitrogen.
NADH	:	Reduced form of nicotinamide adenine dinucleotide.
ONPG	:	:o-nitrophenyl- β -D-galactopyranoside.
PMS	:	Phenazine metasulphate.
pNPP	:	4-nitrophenyl disodium orthophosphate.
ppGpp	:	Guanosine-3'-diphosphate-5'-diphosphate.
r.p.m.	:	Revolutions per minute.
RCC	:	Respiring cell count.
RNA	:	Ribonucleic acid.
RNase	:	Ribonuclease
SDS	:	Sodium dodecyl sulphate.
SDS-PAGE	:	Sodium dodecyl sulphate polyacrylamide gel electrophoresis.
SS	:	Synthetic sewage
t_{90}	:	One log drop from initial inoculum size.
TDC	:	Total direct count.
TEMED	:	Tetramethylethyl-enediamine.
Tris	:	Tris(hydroxymethyl)aminoethane.
UV.	:	Ultra-violet.
V	:	Volt
VC	:	Viable count

v	:	Volume
wt	:	Weight.

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1. General Introduction

1.1 General characteristics of *Escherichia coli*

Escherichia coli is a Gram-negative bacterium of the family *Enterobacteriaceae*. *E. coli* is a rod shaped facultative anaerobe which can be cultivated on a simple growth medium employing glucose or glycerol as sole carbon and energy source (Cruickshank *et al.*, 1973, 1975). *E. coli* does not produce a spore. It has peritrichous flagella for motility and usually possesses fimbriae for attachment. *E. coli* produce red colonies on MacConkey's agar because of its ability to ferment lactose (Cruickshank *et al.*, 1973). The shape of a colony of *E. coli* is circular, smooth with an entire edge on solid agar plates, however some strains produce mucoid colonies (Macone *et al.*, 1981). *E. coli* is a faecal coliform which originates primarily in the gastro-intestinal tracks of mammals.

1.2 The role of *Escherichia coli* as an indicator of faecal pollution

Surface water resources such as rivers, lakes and streams are consumed for the purpose of drinking, recreational activities and irrigation. Such natural water sources are contaminated with domestic, agricultural and industrial wastes. When water is polluted with faecal material from human and other warm blooded animals, the opportunity exists for pathogenic microorganisms which periodically occur in the intestinal track to enter the water. White and Godfree (1985) suggested that sewage effluent is the main source of faecal coliforms in estuaries and rivers. Pathogenic microorganisms are found in all rivers where the same water is used for bathing, drinking and the disposal of sewage effluent. Many enteropathogenic diseases are transmitted through water and designated as waterborne diseases (Singh and McFeters, 1992). It is estimated that more than 200,000 children, especially under 5 years old, are

infected in the United States each year with waterborne diarrhoea at an annual cost of \$ 1 billion dollar (Ho *et al.*, 1988). Statistics show that there are 250 million new waterborne cases of diarrhoea causing 10 million deaths worldwide per annum (Synder and Merson, 1982). These observations show the global importance of waterborne disease. It is necessary to understand the properties of indicator organisms in aquatic environments which indirectly allow us to acknowledge the presence of pathogenic bacteria and their possible effect on human health without in fact isolating them.

The commonest diseases originating from watercourses are caused by *Shigella*, *Salmonella*, enteropathogenic *E. coli*, *Campylobacter jejuni*, *Vibrio cholerae*, viruses and protozoa (Singh and McFeters, 1992). Therefore, water can put human life in danger, since it is a potential carrier of pathogenic microorganisms. The causative organisms of many diseases exist in the faeces or urine of an infected person and when discharged may enter a water body which ultimately serves as a source of drinking water (Pelczar *et al.*, 1981).

Some of the bacteria which have been used as indicators of faecal pollution in drinking surface and ground water are *E.coli*, total coliforms (mainly *Citrobacter*, *Enterobacter*, *Klebsiella* sp), *Enterococcus faecalis*, *Enterococcus faecium*, *Enterococcus duran*, *Enterococcus equi*, *Clostridium perfringens*, *Bifidobacterium* sp. and *Pseudomonas aeruginosa* (Hutchinson and Ridgway, 1977). Indicator organisms are microorganisms whose presence in water is evidence that the water is contaminated with faecal material from humans or other warm-blooded animals (Pelczar *et al.*, 1981). Different standards for acceptable numbers of faecal coliforms and *E.coli* in water are expressed on the premise that *E.coli* survives in that habitat for a longer period of time than pathogenic bacteria therefore the absence of *E.coli* from a sample can be taken as an indication that other pathogenic bacteria are also absent (Geldreich, 1970). Finally, there is a potential danger with the presence of enteric bacteria even of low pathogenicity in water.

The reason for preferring coliforms especially *E.coli*, as indicator organisms are practical. They also occur in larger numbers than pathogenic bacteria in the faeces of normal healthy individuals (Flint, personal communication). The detection of *E.coli* and other coliforms is easy and simple. Therefore these bacteria are accepted as the most reliable indicators of faecal pollution (Pelczar *et al.*, 1981).

1.3 Viable but non-culturable bacteria in natural aquatic environments

The survival of bacteria in aquatic environments is known to be influenced by a number of parameters including starvation, heavy metal concentration, salinity, nutrient availability, temperature and injury by disinfectants. Many aquatic bacteria have been shown to enter a natural dormant state when subjected to certain environmental stresses. The viable but non-culturable state or dormancy in bacteria has been defined as a temporary loss of viability (Henis, 1987). Although bacteria may lose capacity to grow in an environment when they enter the viable but non-culturable state, they may continue to take up nutrients, respire and to keep pool of metabolites against the diffusion gradient across the cell membrane (Henis, 1987). The viable but non-culturable phenomenon in bacterial cells was reported first by Jannasch (1967) who showed that in continuous culture, marine bacteria would not divide in seawater even with sufficient nutrients. He suggested that below certain levels of substrate, bacteria were surviving but remained in an inactive state. Kurath and Morita (1983) and Roszak and Colwell (1987) suggested that if the cells had the ability to produce colonies on agar plates containing carbon and energy sources then they should be referred to as viable cells. If they do not form colonies they are not necessarily dead or inactive.

There has always been a difference between cell numbers obtained from direct count and traditional culture methods (Pickup, 1991). For instance Jones (1977) showed that although the potential number of cells in lake water was 10^6

cfu/ml according to a direct count, approximately 10^3 cfu/ml of those were detected as culturable on agar plates. Moreover, studies by Hoppe (1978) concluded that the rate of culturable cells in the marine environment was only 0.01-12.5% of the total viable cells. On the contrary, some bacteria have shown to become non-culturable but retain their viability after exposure to their environment. Many cells which have lost the ability to form colonies on standard culture media, are still viable by direct count methods. Many microorganisms in the aquatic environment remain viable but non-culturable but this does not indicate that these cells are dead, they may be sublethally injured and, therefore, cannot be recovered by the selected media normally used.

It is widely accepted that the stressed cells become more sensitive to inhibitory agents in selective media and therefore cannot grow and produce colonies (Bissonnette *et al.*, 1975; Xu *et al.*, 1982; Grimes and Colwell, 1986). For this reason the use of selective media for the detection of stressed bacteria is an important problem. Therefore there is a discrepancy in the recovery of microorganisms, when selective media or other methods for survival of indicator or pathogenic microorganisms are used (Roszak *et al.*, 1984; Grimes and Colwell, 1986).

Some Gram-negative bacteria such as *Vibrio vulnificus*, *E. coli*, *V. cholerae*, *Salmonella enteritidis* (Roszak *et al.*, 1984; Linder and Oliver, 1989; Kjelleberg *et al.*, 1987), *Shigella sonnei*, *Shigella flexneri* (Colwell *et al.*, 1985) and *Campylobacter jejuni* (Rollins and Colwell, 1986) remain viable but become non-culturable under different stress conditions. This response appears to be a survival strategy of bacterial cells so as to maintain their viability in natural environments. Bacteria use endogenous energy sources in the absence of external nutrients in aquatic environment (Dawes, 1976; Henis, 1987). Cellular components are utilised as endogenous substrates for energy requirements and maintaining the integrity and activity of essential cell components, especially the cytoplasmic membrane (Henis, 1987). Bacterial cells maintain some of essential

nutrients at minimal concentrations for the aim of protecting viability. When the cells enter a dormant state some morphological and physiological changes occur such as cell size reduction, reduction in enzyme activity and reduction in protein synthesis (Morita, 1982; Reeve *et al.*, 1984; Smigielsky *et al.*, 1989). Jones *et al.* (1991) showed that long-term starvation exposure of *C. jejuni* changed its shape from a rod to coccoid form but that Campylobacter antigen remained detectable in the non-culturable cell (Sutcliffe *et al.*, 1991). Similar cell size alterations were detected in *Vibrio* sp under starvation stress in seawater by Kjelleberg *et al.* (1983); and Smigielsky *et al.* (1989).

Dormancy, in other words viability but non-culturability or temporary loss of viability, is a great problem for the detection of faecal coliform bacteria in aquatic systems. Plating methods for estimating the number of indicator organisms such as *E. coli* and waterborne pathogens including *V. cholerae* are of limited use when used to estimate populations of viable but non-culturable organisms in the aquatic environments (Xu *et al.*, 1982). They showed that both *E. coli* and *V. cholerae* disappeared according to plate count however they remained viable according to direct microscopical examination method. Under environmental stress bacteria maintain their virulence and pathogenic capability (Grimes and Colwell, 1986). In addition, Oliver *et al.* (1991) and Oliver and Wanucha (1989) demonstrated that the bacterial pathogen *V. vulnificus* in artificial seawater became viable but non-culturable at 5°C. This kind of dormancy was detected as temperature dependent even at increased nutrient concentration. These viable but non-culturable cells were not able to revive in the rich medium normally used for growth of the organisms. Nilsson *et al.* (1991) concluded that non-culturable cells were not able to grow on plates solid media nor in liquid media. Therefore, when the cells have entered a non-culturable state, standard bacteriological culture methods are not satisfactory for the detection of these cells and special resuscitation methods are required. However, recovery of non-culturable cells of *S. enteritidis* has been performed

by adding nutrient media of different strength and incubating the samples (Roszak *et al.*, 1984). Grimes and Colwell (1986) showed that viable but non-culturable enteropathogenic *Escherichia coli* could be recovered by inoculation into a ligated rabbit ileal loop. These results suggest that waterborne pathogens remained viable but non-culturable and could be revived again in animal passage (Colwell *et al.*, 1989).

All the above suggest that the viable but non-culturable phenomenon is a vital problem for the safety of drinking, recreational and irrigational water in terms of human health aspect. Because faecal pollution indicator organisms may not appear on agar plates but exist in the aquatic system. This causes an underestimation of pathogenic bacteria of faecal origin and leads to the use of contaminated water. If this contains potential pathogens then this will lead to an increase in waterborne diseases. Chlorination is applied as the last step for disinfection of the drinking water so as to remove any remaining micro-organisms and to ensure its potability (Pelczar *et al.*, 1981). However many researchers have noted that chlorination does not kill all the organisms in water, some of them remained injured and could recover again their pathogenic potential (LeChevallier and McFeters, 1985). More detailed studies performed by Stuart *et al.* (1977), Bissonnette *et al.* (1977) and Camper and McFeters (1979) have reported that almost 90% of *E. coli* populations were found to be injured and remained viable but non-culturable by using endogenous energy reserves until recovery occurred. On the other hand, 10% of the bacterial cells could grow on selective media. They also suggested that the concentration of chlorine addition was also important in determining the number of cells injured in a water sample. Chlorine concentrations that resulted in 90% injury of coliforms were between 0.25 and 0.5 mg/L; the level required for comparable injury to pathogens was 0.9 to 1.5 mg/L. Pathogens such as *Yersinia enterocolitica*, *Salmonella typhimurium* and *Shigella* spp. required significantly higher levels of stressors to cause the same level of injury than *E. coli*

(LeChevallier *et al.*, 1985). McFeters and Singh (1991) concluded that the enteric pathogens examined were less susceptible than coliforms from sublethal levels of chlorine.

Singh *et al.* (1986) showed that after chlorination treatment similar to that applied to drinking water enteropathogenic *E. coli* ceased to grow but demonstrated virulence in the mammalian gut. Bissonnette *et al.* (1975) also showed that bacteria injured in natural waters have a prolonged lag phase prior to active growth. Chlorine-injured enteropathogenic *E. coli* cells were able to recover *in vitro* in a saline solution containing a homogenate of mouse intestinal mucosa, but not in the controls containing only saline solution. These results infer that conditions in the mammalian gut are conducive to the repair of enteropathogenic *E. coli* stressed by the addition of chlorine.

In brief enteric bacteria including both indicator organisms and pathogens are affected by different stress conditions in aquatic systems. In case of indicator bacteria, this situation may result in a significant underestimation of the actual number of viable organisms present if commonly used media and method are applied (McFeters and Singh, 1991). Injured waterborne enteric pathogens are a vital important matter in terms of the public health significance. A range of pathogens have been examined and found to retain the ability to cause disease following injury. Injury rarely reduces their ability to cause disease (McFeters and Singh, 1991).

1.4 Factors affecting survival of *Escherichia coli* in aquatic environments

1.4.1 Solar radiation

The bactericidal effect of visible and near-visible light on bacteria was first reported by Hollaender (1943); Gameson and Saxon (1967) suggested that the effect was lethal to *E. coli* cells and this effect was severe when the cells were incubated in starvation medium. This findings were supported by the work of Chamberlain and Mitchell (1978) who also proposed that under field

conditions, ultraviolet (uv) light was a significant stress on bacterial survival in marine environment. The susceptibility of *E. coli* to photoinactivation has also been shown by Lessard and Sieburth (1983). In addition, supportive evidence was provided by McCambridge and McMeekin (1981) for estuarine environments who showed that solar radiation led to decline number of *E. coli* in estuarine water. Fujioka and Narikawa (1982) noted that when faecal coliform and faecal streptococci were subjected to sunlight exposure, 90% of faecal coliform and faecal streptococci were inactivated within 13 to 32 min. The bactericidal effect of sunlight was shown to be able to penetrate both glass and polyethylene containers resulting in the inactivation of 90% of the populations of faecal coliforms in a mixture of sewage and seawater after 13 to 17 min of exposure to sunlight. Gameson and Gould (1975) and Camberlain and Mitchell (1978) reported that sunlight was able to penetrate up to 3.3 m into natural clear seawater. However this effect was reduced drastically in turbid waters such as inland rivers containing sewage effluent. Kapuscinsky and Mitchell (1981) suggested that solar radiation led to the death of bacterial cells due to inactivation of the catalase system through the build up of hydrogen peroxide. McCambridge and McMeekin (1981) and Barcina *et al.* (1986) concluded that solar radiation was an important factor for bacterial survival in aquatic systems, because *E. coli* survival was affected by solar radiation more than biotic factors such as viruses, protozoa, spores, and some pathogenic bacteria. Barcina *et al.* (1989) and Davies and Evison (1991) showed that *E. coli* cells exposed to sunlight could become viable but non-culturable but still detectable by means of metabolic activity measurement with INT reduction activity and acridine orange direct count (AODC). In addition, the light source is also an important factor on bacterial survival (Davies and Evison, 1991). According to their findings, there was a rapid increase in the number of injured *E. coli* subjected to natural sunlight compared to a similar intensity artificial light source. The decline in the number of *E. coli* occurred through the effect of both the UV components of sunlight and

the high salinity. They also showed that a decrease in glucose uptake by *E. coli* occurred under the visible light condition. As a consequence, they concluded that visible light was also responsible for an additional inhibition of biosynthetic processes.

1.4.2 Nutrient deficiency

In aquatic environments, the survival of bacteria is strongly dependent on the presence of nutrient sources. Nutrients allow the bacterial cells to build up necessary material for cell biomass and for the production of biologically utilizable energy and are required for some functions such as cellular motility (Dills *et al.*, 1980). It has been reported by several researchers that the addition of organic and inorganic nutrient supplements to water enables coliform bacteria to reduce their rate of disappearance from the aquatic environments (Carlucci and Pramer, 1960; Lim and Flint, 1989). Carlucci *et al.* (1986) showed that only 15% of the bacteria capable of growing on a rich medium were able to grow on unsupplemented seawater agar. Chai (1983) noted that nutrient supplementation of natural waters delayed the reduction in the number of viable cells but numbers did decrease even though direct count revealed can increase in count. He also demonstrated that rich medium grown cells survived for a longer period than those grown in a basal medium. Flint (1987) pointed out that *E. coli* could grow better in a sewage-polluted river water than at less polluted site.

In natural aquatic environments most bacteria exist under nutrient-limited conditions. Therefore microbial growth in natural aquatic environment must occur only under the stress of starvation in most aquatic environments (Veldkamp and Jannasch, 1972; Tempest and Neijssel, 1981).

There have been many reports of starvation-induced changes in bacterial cells in aquatic environments (Dawes, 1976; Morita, 1982; Kjelleberg *et al.*, 1987). These changes appear as morphological and physiological alterations in bacterial cells (Morita, 1982; Kjelleberg *et al.*, 1982). A significant reduction in

cell size is generally accepted as a general phenomenon when cells are subjected to nutrient deprivation (Novitsky and Morita, 1977; Smigielsky *et al.*, 1989, 1990). Such morphological changes in cell size has been termed 'miniaturisation' (Morita, 1982). MacDonell and Hood (1982) showed that *Vibrio*, *Pseudomonas*, *Aeromonas* and *Alcaligenes* spp. were found as very small cells and considered this to be an adaptation to the nutrient-poor environment.

Many bacteria produce specialised forms such as endospores of *Bacillus* spp and the exospore of *Streptomyces* spp. in response to starvation stress. Non-spore forming bacteria can also survive for a long-time under conditions of nutrient depletion by using endogenous energy supplies, reducing cell size ratio and having a low metabolic activity (Morita, 1982). Flint (1987) reported that *E.coli* could survive for 260 d in river water without the addition of any nutrient sources at 25°C when the natural microbial flora had been eliminated by autoclaving. To survive for this period of time without addition of any nutrient *E.coli* must enter a dormant state similar to that described by Morita and his co-workers for a marine *Vibrio* under nutrient-limited conditions (Morita, 1982).

Extreme starvation stress on bacteria including *E.coli* causes cells to enter a dormant state, that is they can become viable but non-culturable which is very important for the detection of indicator bacteria (Novitsky and Morita, 1977; Dawson *et al.*, 1981; Colwell *et al.*, 1985). This phenomenon in *E.coli* and *S. typhimurium* examined in water by Roszak and Colwell (1987) who showed that these bacteria were not able to grow on standard culture media but the cells were detectable by direct viable count techniques.

Other common alterations in starved bacterial cells include an increased tendency for adhesion and change in cell hydrophobicity. This might be important during substrate changes (Dawson *et al.*, 1981; Kjelleberg and Hermansson, 1984; 1987). Because surface-active organic molecules are accumulated at surfaces (Hunter, 1980) these nutrients can be utilised by

surface-attached bacteria (Hermansson and Marshall, 1985). Dawson *et al.* (1981) showed that during the initial phase of starvation some fimbriae were formed by the marine *Vibrio* DW1 and starvation increased adhesion to a glass surface. Kjelleberg and Hermansson (1984) found that the degree of hydrophobicity of cells was increased in *Vibrio* by starvation stress and suggested that this was due to increased fimbriation or due to change in other cell surface components such as lipopolysaccharide.

Some physiological and metabolic alterations have been investigated, mostly in marine bacteria in response to starvation stress. Generally these alterations occur in transport mechanisms and nutrient uptake, the proton motive force, the stringent response, the degradation of reserve polymers and endogenous macromolecules, the release of catabolite repression which may be required in order that different substrates from the environment may be utilised (Kjelleberg and Hermansson, 1987). Copiotrophic bacteria must undergo a number of physiological and metabolic adaptations in the nutrient-depleted environments in order to meet their energy requirements and improve their chances of long-term survival. These include a low respiration rate (Novitsky and Morita, 1977), the regulation and induction of starvation specific proteins (Reeve *et al.*, 1984; Groat and Matin, 1986; Nyström *et al.*, 1986; Jouper-Jaan *et al.*, 1986) and the use of endogenous reserve material (Jones and Rhodes-Roberts, 1981). Furthermore the ability to scavenge nutrients efficiently from the environment and transport them into the cells are also important under nutrient-limited conditions (Morita, 1984). The most significant changes occur during the initial transient phase of starvation. These include a rapid decline in the total amount of carbohydrate and lipid (Hood *et al.*, 1986) and polyhydroxybutyrate (Malmcrona-Friberg *et al.*, 1986) in the cell, an increase in the ratio of unsaturated to saturated fatty acids in the membrane which may increase fluidity of the membrane (Malmcrona-Friberg *et al.*, 1986).

and a temporary increase in amino acid uptake and incorporation (Nyström *et al.*, 1986).

1.4.3 Temperature

Temperature is one of the important environmental factors influencing survival of coliform bacteria in aquatic environments. Growth temperature can significantly influence the physiological state of bacterial cells (Neidhardt *et al.*, 1990). Temperature may affect both the growth rate and the yield of bacteria (Harrison and Loveless, 1971) and also influence respiration rate. Barcina *et al.* (1986) showed that *E.coli* survival was longer at 10°C than 28°C due to lower metabolic activity in river water. In addition, Flint (1987) suggested that temperature was one of the major factors for the disappearance of *E.coli* from river water. He showed that whilst *E.coli* survived the longest at 4°C and shortest at 37°C, the presence or absence of natural microbial flora was the most important reason for the declining in cell number. He also showed that with the absence of natural competitors *E.coli* was able to survive 260 days at temperature lower than 25°C in unamended river water.

Increasing temperature above the normal range leads to heat shock response in the cells. In *E.coli* 24 proteins are induced due to an increase in temperature, 20 of which are under the control of a single gene *htrR*, which encodes a protein sigma factor (Neidhardt *et al.*, 1990). Lim (1988) showed that *E.coli* cells disappeared after heat shock treatment for a few days then seemed to reappear on agar plates in lake water microcosm. This was probably due to repair of damage caused by heat shock.

However temperature itself probably is not an important factor for the survival of *E.coli* in the aquatic environment. The organism is not killed or injured by temperatures below 45°C. The effect of temperature on survival are due to the influence which temperature has on other biological functions. For instance predators are more active at 30°C than at 4°C hence survival would be

expected to be longer at 4°C. Similarly *E. coli* is more active at 30°C than at 4°C hence more nutrients would be needed to support *E. coli* survival at 30°C than 4°C. The surprising result is that Flint (1987) was able to show long-term survival in the absence of the natural microflora at 25°C, a temperature at which *E. coli* is metabolically active. The only conclusion here is that there was sufficient nutrient available to enable the cells to enter a dormant state, this was not possible if the natural microflora was available to also compete for the available nutrient.

1.4.4 Predation

Bacteria can be eliminated from the aquatic environment by predation from protozoa, or by other organisms for example predatory bacteria (e.g. *Bdellovibrio* spp) and bacteriophages (Gurijala and Alexander, 1990; González *et al.* 1992). González *et al.* (1992) suggested that among these factors protozoan grazing was the main reason for the decline in the total number of enteric bacteria in aquatic environments. They also found that the number of *E. coli* and *E. faecalis* in both freshwater and seawater samples declined due to protozoan predation. Others have also shown that predation by protozoa leads to a decline in the number of *E. coli* in river, estuarine and lake water (Barcina *et al.*, 1986; Rhodes and Kator, 1988). Using metabolic inhibitors and Millipore filtration to selectively eliminate eukaryotic cells, it is possible to estimate the effect of predation on bacterial survival in aquatic environments. According to these findings, the relation between the presence of protozoa and coliform destruction was supported when estuarine water was filtered through membranes of different size (McCambridge and McMeekin, 1981). Less coliform destruction occurred in water if protozoa were removed from estuarine water (Enzinger and Cooper, 1976). Similar results were obtained by Sorensen (1991) who showed that *E. coli* was eliminated by protozoa in estuarine water. The addition of eukaryotic inhibitors such as cycloheximide and nystatin led to a longer survival

for *E.coli* in water. Protozoa could not be detected in the sample after a day while *E.coli* cells were still present for up to 15 days. Predation by protozoa of coliform bacteria was also reported by Mallory *et al.* (1983) who showed that the disappearance of *S.typhimurium* and *Klebsiella pneumoniae* from sewage was due to predation. On the other hand, Sanders and Porter (1986) suggested that protozoans and several ciliates were not inhibited by cycloheximide. In river water microcosms, Flint (1987) showed that there was a little difference in survival times of *E.coli* in untreated and Whatman-filtered lake water samples. Whatman-filtered lake water did not contain protozoa. However when a river water sample was autoclaved *E.coli* survival was prolonged due to the removal of effective competitors or prevention of predation by bacteriophage which may have been responsible for the difference in survival times. This conclusion was supported by Bergh *et al.* (1989) and Proctor and Fuhrman (1990) who reported that bacteriophages are very abundant in aquatic environment and these organisms could play a significant role in bacterial mortality.

1.4.5 Osmolarity

Osmolarity is an important factor affecting growth and survival of enteric bacteria when they are released to a salty aquatic environment (Munro *et al.*, 1989). Cells exposed to hypertonic solutions will respond by dehydration of the protoplast. However, bacteria and other *Enterobacteriaceae* have the ability to overcome this osmotic pressure through an osmoregulation process which leads to the accumulation of potassium ions and consequently reduced cell turgor (Epstein, 1986). In addition several members of the *Enterobacteriaceae* take up molecular organic solutes such as amino acids, polyhydric alcohols and betaines (N-methylated amino acids derivatives) in order to tolerate osmotic stress (LeRudulier *et al.*, 1984b) and to build up the internal pressure to prevent diffusion of water out of the cell. Gauthier *et al.* (1987) showed that when *E.coli* was preadapted to high osmolarity in salt medium, survival was

increased. Osmoprotectants is the general term for organic solutes which can accumulate in the cells in large amounts and protect cells against osmotic stress and salt inactivation (LeRudulier *et al.* 1984a). Measures (1975) showed that amino acids especially proline, stimulated growth and respiration of some bacteria which accumulated it in response to increased sodium chloride concentration. Glycine betaine (N,N,N-trimethylglycine) is known to be the best osmoprotectants accumulated in the cells under the conditions of hyperosmolarity.

1.5. Physiological alterations in bacterial cells under different stress conditions

1.5.1. The changes in protein patterns

The alterations in protein synthesis under different stress conditions may be important for the retention of cell viability during metabolic stress. In many environments, bacteria are starved for energy, carbon, nitrogen or phosphorus sources and the absence of these essential requirements results in alterations in protein synthesis. There are some proteins in bacterial cells which are only synthesised under the stress of heat, ethanol, hydrogen peroxide, osmotic pressure and starvation stress in aquatic environments (Hecker and Völker, 1990).

It has been reported that starvation specific proteins enable cells to improve their ability to efficiently find and scavenge substrates. It was proposed that starvation-specific induced proteins are related to increased adhesion (Kjelleberg *et al.*, 1987), altered chemotaxis (Morita, 1985), improved uptake capacity i.e. switches from low to high affinity uptake systems for amino acids and sugars (Morita, 1985; Mårdén *et al.*, 1987) and the synthesis and excretion of exoproteases (Nyström *et al.*, 1989). In addition, Reeve *et al.* (1984) showed by using peptidase-deficient mutants of *E.coli* that during starvation, the

degradation of proteins is used to provide essential amino acids for the synthesis of new proteins. It has been shown by Horan *et al.* (1981) that amino acids obtained from the degradation of proteins are used as endogenous substrate. Kjelleberg *et al.* (1987) suggested that the chance of bacteria surviving under starvation stress is increased by the production of new proteins but he concluded that these proteins do not seem to be directly related to the viability of the cell during the initiation process.

Most starvation proteins are synthesised at the onset of the starvation process and are necessary for survival for long starvation time periods (Reeve *et al.*, 1984; Groat and Matin, 1986). Using two-dimensional gel electrophoresis, dramatic changes in the protein finger print patterns were detected in *E.coli* (Reeve *et al.*, 1984), in the marine psychophilic *Vibrio* Ant-300 (Amy and Morita, 1983), in *Salmonella* spp (Reeve *et al.*, 1984) and some other bacteria. It has been shown that in *E.coli* at least 30 polypeptides, including cytoplasmic as well as membrane proteins, are induced under succinate and carbon starvation during the first 3-4 h (Groat and Matin, 1986). Schultz *et al.* (1988) showed that starvation for nitrogen, carbon and phosphorus induced up to 32 polypeptides and that some of these proteins were specific for starvation stress. However, 13 of those were synthesised under all three starvation conditions. In addition, Spector *et al.* (1986) reported that 6 common proteins were synthesised under phosphate, ammonium and nicotinate starvation in *S. typhimurium*. These common starvation specific proteins are termed 'Pex proteins' (Matin, 1990).

It has been reported by Matin *et al.* (1990) that guanosine 3'-diphosphate-5'-diphosphate (ppGpp) plays an important role in the regulation of starvation protein synthesis under lack of amino acid conditions which is termed the "stringent response". Nyström *et al.* (1990) showed that the shut-down of RNA, protein and peptidoglycan synthesis occurred in starved bacteria in the first 0-30 min proportional to the accumulation of ppGpp. The importance of the stringent response in starvation survival has been demonstrated by Mach *et al.* (1989)

who showed that three independent rel A mutants of *E.coli* lost viability significantly faster than their isogenic rel A⁺ counterparts during amino acid starvation.

Osmotic stress is one of the other factors causing new protein synthesis in bacterial cells. Hecker and Völker (1990) explained that salt stress is the best inducer of general stress proteins and also induces some specific proteins other than general stress proteins. Clark and Parker (1984) showed that osmotic pressure affected the protein patterns of *E.coli* and induced a specific group of proteins (Jenkins *et al.*, 1988).

Farr and Kogoma (1991) noted that sublethal concentration of hydrogen peroxide resulted in an increase of 30 oxidation stress proteins in *E.coli*. Although starvation, heat shock and hydrogen peroxide each produces its own individual protein patterns, several starvation proteins were common to either heat or oxidation stress and few were common to both (Jenkins *et al.*, 1988). In addition, sodium dodecyl sulphate (SDS) addition to the growing medium lead to the induction and repression of some proteins in *E.coli* (Adamowicz *et al.*, 1991) and in *Enterobacter cloacae* (Kramer and Nickerson., 1984). The detergent stress stimulon appears to be independent from the heat shock stimulon and the oxidation stress stimulon. Adamowicz *et al.* (1991) detected that 5 proteins appeared, 4 proteins disappeared in *E.coli* and also 15 proteins were elevated and 15 proteins were repressed in growing medium to which 5% SDS had been added.

1.6 Aims

The aims of this project were;

1. To investigate the survival of *E.coli* ML30 in lake water under the effect of different stress conditions and environmental parameters. The stress factors studied included starvation, incubation temperature, oxidation stress, detergent stress, alcohol and chlorine stress.
2. To assess the effects of nutrients and inhibitors on the survival of *E.coli* ML30 in the presence and absence of the natural microflora in lake water microcosms.
3. To investigate the effects of synthetic sewage amendment on *E.coli* ML30 survival in lake water in the presence and absence of natural microflora in lake water microcosms.
4. To assess the activity of some enzymes of *E.coli* ML30 under long-term starvation stress and different nutrient conditions in lake water microcosms and to establish a possible link between enzyme activity and survival. Those enzymes studied included respiratory enzymes, alkaline and acid phosphatases and 8-galactosidase activity.
5. To investigate the non-culturable stage of *E.coli* cells in lake water microcosms using a number of techniques to determine viable and direct counts..
6. To determine the effects of long-term starvation on the physiological status of *E.coli* ML30 in lake water microcosms by changes in protein patterns by SDS-PAGE and two-dimensional gel electrophoresis.

Chapter II. Materials and Methods

2.1 Strain

Escherichia coli ML30 was used for all the experiments. It was provided from University of Warwick culture collection (K.P.Flint). The organism was obtained originally from Queen Elizabeth College, London. The organism was sub-cultured every four weeks onto nutrient agar plates grown at 30°C and stored at 4°C.

2.2 Media

2.2.1 Nutrient Agar

Most viable counts were performed on nutrient agar and stock cultures were also maintained on nutrient agar. 28 g of nutrient agar (Oxoid, UK) were dissolved in 1 litre of double distilled water, sterilised at 121°C for 15 min and poured into 9 cm diameter plastic petri dishes (Sterilin Ltd., UK). The plates were dried in an air-flow cabinet at room temperature and stored at 4°C until used.

2.2.2 Eosin-Methylene Blue Agar (EMB)

EMB was used for the detection of *E.coli* in water samples. EMB plates were made according to the manufacturers instructions.

2.2.3 Minimal Medium

Minimal medium was used as a growth medium in the alkaline phosphatase enzyme experiments.

The minimal medium was made by mixing the following quantities of sterile stock solutions:

Tris buffer,	(12.1 g/L)	500 ml
MgSO ₄ 7H ₂ O	(10 g/L)	10 ml
(NH ₄) ₂ SO ₄	(10 g/L)	100 ml
CaCl ₂	(1 g/L)	100 ml
FeSO ₄ 7H ₂ O	(0.05 g/L)	10 ml
Trace Element Solution		2.5 ml

The trace element solution was prepared according to protocol of Kelly and Clarke (1962) as follows:

(NH ₄) ₂ FeSO ₄ .6H ₂ O	232 mg
H ₃ BO ₃	464 mg
CoSO ₄ .7H ₂ O	191 mg
CuSO ₄ .5H ₂ O	16 mg
MnSO ₄ .4H ₂ O	16 mg
(NH ₄) ₆ Mo ₇ O ₂₄ .H ₂ O	44 mg
ZnSO ₄ .7H ₂ O	348 mg

The trace elements were dissolved in 1 litre double distilled water and 1 M HCl was added to solution dropwise until the solution cleared.

The pH was adjusted to 7.2 with 0.1 N HCl. The volume was made up to 800 ml with distilled water. 80 ml of this solution was dispensed into 250 ml Erlenmeyer flasks. All the flasks were sterilised by autoclaving at 121°C for 15 min. Afterwards each of these flasks was supplemented with carbon and phosphorus sources as below;

a) Carbon source: Succinate was added to give a final concentration of 0.5 g-C/L. 10 ml of a stock solution of sodium succinate (28.2 g/L), sterilized at 121°C for 15 min was added aseptically to 80 ml of the minimal medium.

b) Phosphorus source: 1 g PO_4/L solution; 1.431 g KH_2PO_4 was dissolved in 1 L of distilled water and sterilised by autoclaving at 121°C for 15 mins. High phosphate minimal medium contained 10 ml of this stock solution in a final volume of 100 ml. Low phosphate minimal was made by adding 1 ml of the stock and 9 ml of distilled water to the mineral salts medium described above.

2.2.3.2 M9 Mineral Salts Medium

M9 mineral salts (Lark *et al.*, 1963) was used for nitrogen-limited starvation survival experiments.

M9 mineral salts was made up in 1 litre double distilled water as follows;

$\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$	7.0 g
KH_2PO_4	3.0 g
NaCl	0.5 g
NH_4Cl	1.0 g
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.2 mg
CaCl_2	20 mg

The pH was adjusted to 7.0 and the medium sterilised by autoclaving at 121°C for 15 mins. Glucose was added aseptically at a final concentration of 2 g-C/L to this above solution as carbon source. The glucose solution was sterilized by autoclaving at 121°C for 15 min. For nitrogen-limited minimal medium, ammonium chloride was added to the solution at final concentration 0.01 g/L instead of 1.0 g.

2.2.3.3 Basal Medium for β -galactosidase enzyme experiments

The medium of Dobrogoz (1981) was used as the growth medium for the induction of β -galactosidase.

Following concentrations were added to 1 litre of double distilled water:

KH_2PO_4	2 g
K_2HPO_4	7 g
$(\text{NH}_4)_2\text{SO}_4$	1 g
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$	0.1
Vitamin-free casein hydrolysate	2.5 g

The pH was adjusted to 7.2 and the medium sterilized at 121°C for 15 min.

Carbon source; 1 g-C/L final concentration of glycerol was added after sterilisation at 121°C for 15 min. Also 2.5 mM final concentration of isopropyl- β -D- thiogalactopyranoside was added as inducer of β -galactosidase.

2.2.4 Nutrient Broth Medium

Nutrient broth was employed for culturing of *Escherichia coli* to prepare the inocula for the survival experiments. 1.3 g nutrient broth powder (Oxoid, UK) was dissolved in 100 ml of double distilled water in a 250 ml Erlenmeyer flask. These flasks were sterilised by autoclaving at 121°C for 15 min and stored at 4°C until required.

2.2.5 Quarter Strength Ringer Solution

Normal strength Ringer solution consisted of the following 1 litre of double distilled water.

NaCl	9.0 g
CaCl ₂	0.48 g
NaHPO ₄	0.2 g
KCl	0.42

Normal strength Ringer solution was sterilised by autoclaving at 121°C for 15 min and kept as stock solution at 4°C. This solution was diluted to quarter strength and used to prepare the serial dilution for the viable counts in the survival experiments.

2.3 Water Samples

Lake water samples were collected in 2 litre capacity sterile glass bottles from Tocil Lake which is located on the University of Warwick Campus (OS ref. 303756). This lake is a man-made lake, populated by wild fowl.

Lake water samples were collected fresh for every experiment especially for experiments with untreated lake water, because the age of the lake water may cause the decay of some molecular structures and also lead to the elimination of some microorganisms due to starvation. As a consequence this can affect the experimental data. Filtered-autoclaved lake water samples were used in some survival experiments. These were filtered through Whatman No-1 (Whatman) filter paper and autoclaved at 121°C for 15 or 20 min.

For the competition experiments, lake water samples were filtered through Whatman filter paper, 0.45 µm Millipore or 0.2 µm Millipore filters prior to experiments.

2.4 Nutrient Sources Amendments

The lake water samples were, in some cases, amended with some carbon sources, nitrogen sources, amino acids or inhibitors. All the amendments were

sterilised by autoclaving at 121°C for 15 min. and then added to the lake water. The concentration of nutrient sources additions were shown in the figure legends.

2.4.1 Synthetic Sewage

Various concentrations of synthetic sewage were added to the lake water. Synthetic sewage was made as follows;

Yeast (Oxoid)	0.3 g
Peptone (Oxoid)	0.2 g
Urea	0.05 g
(NH ₄) ₂ SO ₄	1.0 g
KH ₂ PO ₄	0.2 g
FeSO ₄ .7H ₂ O	1 mg
Trace element solution	5 ml

This mixture was dissolved in 1 litre of double distilled water and autoclaved at 121°C for 15 mins. The trace element solution was added to this mixture is described in 2.2.3.1.

2.4.2 Carbohydrate amendments

Different concentration of carbohydrate sources were added to the lake water. All the carbon sources were prepared in double distilled water and sterilised by autoclaving at 121°C for 15 min.

Carbohydrates used as amendments were as follows;

All carbon sources obtained from BDH. Sucrose, D-glucose, DL-lactose, D-fructose, D-galactose, mannose and maltose were used in the different

experiments at final concentration of 1.0 to 6.0 mg-C/L in lake water microcosms.

2.4.3 Sugar Alcohol Amendments

Between 1.0 to 6 mg-C/L final concentrations of sugar alcohols were added to the 100 ml lake water microcosms in the survival experiments. All the sugar alcohols were dissolved in distilled water then sterilised by autoclaving at 121°C for 15 min. The sugar alcohols used were;

Glycerol (Prolab) and mannitol (BDH).

2.4.4 Carboxylic Acids Amendments

All the carboxylic acids were dissolved in double distilled water and sterilized by autoclaving at 121°C for 15 min. The maximum final concentration of carboxylic acid used was 6 mg-C/L. The carboxylic acids used were;

sodium acetate (BDH), sodium formate (M&B), sodium lactate (Fisions), sodium fumarate (Fisions), sodium propionate (Fisions), sodium citrate (BDH) and sodium pyruvate (BDH).

2.4.5 Amino Acids Amendments

Amino acids were added to the filtered-autoclaved and untreated lake water in survival and enzyme activity experiments. Some of the amino acids were sterilised by filtering the solutions through 0.2 µm Millipore filters instead of autoclaving because of the possibility of denaturation of these amino acids. These were as follows;

L-arginine (BDH)

DL-tryptophan (Sigma)

L-asparagine (BDH)

L-glutamate (BDH)

L-cysteine (Sigma)

L-tyrosine

Some of the amino acids were sterilised by autoclaving at 121°C for 15 min. These were as follow;

L-glutamine(Sigma)

glycine (BDH)

L-leucine(Sigma)

proline (BDH)

L-valine (Sigma)

L-serine (BDH)

L-alanine (Sigma)

L-histidine (Sigma)

L-lysine (Sigma)

L-methionine (Sigma)

L-phenylalanine (Sigma)

2.4.6 Nitrogen Sources Amendments

All the nitrogen sources was used in the survival and enzyme activity experiments were dissolved in double distilled water and sterilised by autoclaving at 121°C for 15 min. prior to addition.

Nitrogen sources were added as follows;

Ammonium sulphate (Anala R), ammonium nitrate (Sigma), casein (Sigma), potassium nitrate (BDH) and sodium nitrate (BDH).

2.4.7 Metabolic Inhibitors

Antibiotics, CCCP (carbonyl cyanide-m-chlorophenylhydrazone) and sodium azide were used as metabolic inhibitors to observe their effect on *E.coli* survival. The antibiotics, cycloheximide, nalidixic acid, streptomycin, spectinomycin, neomycin, kanamycin and ampicillin were dissolved in double distilled water. Nalidixic acid required a few drops of NaOH, and ampicillin required a few drops of sulphuric acid for complete solubilisation to occur. Nystatin was dissolved in ethanol. Stock solutions were 1% (w/v). All the antibiotic solutions were filter sterilised through 0.2 μ m Millipore filter. These antibiotics were added to the filtered-autoclaved and untreated lake water at

different concentration in the survival experiments. All the stock solutions were kept at -21°C.

The respiratory inhibitors, sodium azide and CCCP, were dissolved in double distilled water then were filtered through a 0.2 µm Millipore filter. They were stored at -21°C until used.

2.4.8 Sodium Chloride Amendments

Sodium chloride was added directly to the lake water samples then sterilised at 121°C for 15 min. Final concentrations of sodium chloride additions were between 0.01 to 0.9 M.

2.4.9 Hypochlorite Amendments

Sodium hypochlorite was added to the filtered-autoclaved and untreated lake water samples to give a final concentration between 0.015 and 0.29 M. Sodium hypochlorite solution was filtered through 0.2 µm Millipore filter prior to additions. The stock sodium hypochlorite solution was kept at 4°C until used.

2.5 Survival Experiments

2.5.1 Starvation Experiments

Escherichia coli was grown on nutrient broth overnight at 30°C. Cells were harvested 10 ml of culture by MSE multex centrifuge at 3440 g for 10 min. The cells were washed two times with 10 ml volume of sterile lake water and pellet resuspended in 10 ml of sterile lake water. 100 ml lake water in a 250 ml Erlenmeyer flask was inoculated with 0.1 ml of resuspended culture *E. coli* to give an initial viable count approximately 10^6 cfu/ml. The flask was wrapped in aluminium foil and incubated at the desired temperature in the dark without

shaking. Viable counts were determined on surface-spread nutrient agar plates after overnight incubation at 37°C. The survival experiment samples were usually taken out every day at 30°C and 37°C, every two days at 25°C and 15°C and every three days at 4°C.

2.5.2 Preheated *E. coli* cells

Escherichia coli was grown on nutrient broth or low phosphate minimal medium overnight at 30°C. Cells were harvested 10 ml of culture by centrifugation at 3440 g for 10 min. The cells were washed two times with 10 ml volume of sterile lake water and pellet resuspended in 10 ml of sterile lake water. 100 ml lake water in a 250 ml Erlenmeyer flask was inoculated with 0.1 ml or 0.5 ml of resuspended culture *E. coli* to give an initial viable count approximately 10^6 to 10^7 cfu/ml. These flasks were kept in a water bath at 55°C for either 10 mins for a short-term heat shock experiment or 13 days for long-term experiment for assessing survival.

2.5.3 *Escherichia coli* Pretreated with Detergent

E. coli was grown at 30°C in nutrient broth overnight to which was added 0.5% (w/v) of sodium dodecyl sulphate (SDS), 5 h after inoculation. The cells were harvested, washed with sterile distilled water then resuspended in sterile filtered-autoclaved lake water. 1 ml of SDS-pre-treated cells was added to the filtered-autoclaved lake water for survival experiment. The stock SDS (5% w/v) solution was dissolved in double distilled water and filtered through from 0.2 µm Millipore filter prior to addition.

2.5.4 *Escherichia coli* Pretreated with Ethanol

E. coli was grown overnight at 30°C in nutrient broth to which was added 1% (v/v) ethanol after 5 h of inoculation. The cells harvested, washed with sterile distilled water then resuspended in sterile lake water. 500 ml of filtered-autoclaved lake water was inoculated with 5 ml of ethanol-pre-treated *E. coli* to give an initial viable count of approximately 10^7 to 10^8 cfu/ml.

2.6 Determination of *Escherichia coli* counts

2.6.1 Plate Count (Viable Counts)

The viable counts of *E. coli* were determined by the surface spread-plate technique. 0.5 ml samples were taken from the flasks each sampling time and 10-fold serial dilutions were prepared with quarter-strength Ringer solution. 0.1 ml of the diluted sample was spread on duplicate or triplicate nutrient agar plates. Plates were incubated at 37°C for 18 or 24 h. Plates were counted manually and results were expressed as mean colony forming units per ml.

2.6.2 Respiring and Total Direct Count Examination

2.6.2.1 Prepared solutions

0.2% (w/v) 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT) (Baker Chemical) in double distilled water,

Acridine Orange: 0.01% (w/v) in 6.6 mM phosphate buffer pH 6.7,

Sudan Black B: 6.66 mg Sudan Black B (Merck) was dissolved in 50 ml ethanol then added 50 ml sterile double distilled water.

37% ready stock formaldehyde solution

All above solutions were passed through from 0.2 μm Millipore filter and kept at 4°C until required.

2.6.2.2 Preparation for Microscopical Examination

Polycarbonate filters (Nucleopore corp, 25 mm in diameter, pore size 0.1 μm)

10 ml disposable sterile syringes

Swinnex filter (Millipore)

Oil immersion objective

Immersion oil

Eyepiece micrometer (25 squares with each square 0.0016 mm²)

2.6.2.3 Experimental process

Sample preparation

The method used here was adapted from Zimmerman *et al.* (1978). 10 ml of lake sample was removed from the flasks into sterilized 20 ml plastic universals. 1 ml volume of 0.2% aqueous solution of INT was added samples were incubated at 37°C for 30 minutes in the dark. The reduction of INT was stopped by the addition of 0.1 ml 37% formaldehyde solution to the reaction mixture. This also preserved the sample for up to 1 month with storage at 4°C (Zimmerman *et al.*, 1978).

Microscopical Examination

Polycarbonate filters (Nucleopore 25 mm diameter, pore size 0.1 μm) were immersed in a solution of Sudan Black B for 48 h.

5 ml of the sample was passed through the stained filter which was supported in a Swinnex filter disc (25 mm in diameter, Millipore). Millipore filters were rinsed with double distilled water before the sample was applied. After filtration 0.5 ml of acridine orange solution was dropped onto the filter for

a maximum of 3 min. Filters were dried in the air and cut into four pieces with a sharp razor blade. One piece was covered with immersion oil on a microscope slide and covered with a cover slip. The mounted filter was examined by means of Nikon Optiphot microscopy equipped with an epifluorescent illumination system. The microscope was fitted with a Nikon 100 W mercury lamp, Nikon FX-35A camera with UFX-IIA exposure system and a Nikon DMS 10 FITC filter set. The determination of the total count and cells with formazan spots was carried out at a magnification of 1000X. The total number of bacteria and respiring bacteria were determined in each the square of the eyepiece graticule. Bacteria were counted from 25 random fields from the central point to edge.

2.6.3 Coulter Counter

All counts and size distribution analysis were carried out on a model ZBI Coulter Counter fitted with a Coulter Channelyzer C1000. All the data was stored on a floppy disk using disc drive model CBM 8032 and printed when required. Mean counts were determined from 3 measurements. Latex particles were used as calibration standards.

2.6.4 Electron Microscopy Examination

Solutions

1% w/v Formvar in chloroform solution

1% w/v potassium phosphotungstic acid (PTA) pH 7.0

0.5% w/v Uranyl acetate, pH 4.5

A cover slip was placed in formvar solution and withdrawn immediately in order to form a formvar film and dried in the vapour of the solution. The film was floated off onto the surface of dust-free distilled water. Then a few grids

were placed on the film and picked up with aluminium foil which was allowed to dry at room temperature. A drop of the sample to be examined was placed on the grid for 1 min. Excess liquid was removed using a piece of filter paper. Negative staining was performed with PTA or uranyl acetate. One drop of stain was added and left for a maximum of 30 second. This was removed using filter paper. Finally the grid was observed under transmission electron microscopy. Kodak Panatomic X film were developed in Kodak D19 developer at 20°C for 3 minutes and fixed with Kodafix. Prints were made using Kodak Veribrom paper.

2.7 Enzyme Assays

2.7.1 Alkaline Phosphatase Activity Assay

E. coli was grown in low phosphate minimal medium overnight at 30°C. The cells were harvested and washed with tris-HCl buffer pH 9.0. 100 ml of filtered autoclaved lake water was inoculated with 0.5 or 1 ml of culture to give an initial viable count of approximately 10^6 to 10^7 cfu/ml. The flasks were incubated in the dark without shaking at different temperatures. Alkaline phosphatase activity was measured by removing 2.4 ml volume aliquots, buffering in Tris buffer, pH 9.0 and using pNPP as a colorimetric substrate. The assay was performed at 30°C. Activity was expressed as changes in absorbance at 420 nm/3 h. Total alkaline phosphatase activity was expressed without reference to the different viable count in each microcosm. On the other hand, specific activity refers to the change in absorbance at 420 nm per 3 h per unit of viable count.

Assay Reagents

1 M Tris buffer (Fisons). 121 g Tris dissolved in distilled water buffered to pH 9.0 with HCl. The volume was adjusted to 1 liter and the buffer sterilized by autoclaving at 121°C for 15 min.

0.5% (w/v) 4-nitrophenyl disodium orthophosphate (pNPP) (in distilled water)

10 M NaOH (in distilled water)

Reaction mixture

2.4 ml sample from the flask

0.3 ml 1 M Tris buffer

0.3 ml pNPP

Control (blank sample)

2.4 ml distilled water

0.3 ml Tris buffer

0.3 ml pNPP

The reaction mixtures were incubated in a water bath in sterile bijoux bottles at 30°C for 3 h. The reaction was stopped by the addition of the 0.2 ml of 10 M NaOH. The absorbance was measured at 420 nm using a spectrophotometer (Phillips, PU 8720)

2.7.2 Respiratory Enzyme Assays

2.7.2.1 Electron Transport System Activity

Electron transport system activity (ETS) was measured using the method of Marxsen (1988) and Prin *et al.* (1990). 10 ml samples were added to 20 ml sterile plastic universals and the universals wrapped in aluminium foil and 1 ml 0.2% INT was added. The samples were incubated at the desired for 1 h in a

rich medium or up to 24 h for a starvation medium in the dark without shaking. INT reduction activity was stopped by the addition of 0.1 ml 37% (v/v) formaldehyde. The cells were pelleted in a Beckman J2-21 centrifuge with a JA-17 rotor (13,800 g for 10 min) at 4°C. The supernatant was removed completely from the samples. INT formazan deposits were extracted from the cells in methanol at 4°C for 1-2 h until the pellet was bleached completely. The extract was centrifuged at 13,800 g for 15 min at 4°C to remove cell fragments. The absorbance was recorded on a spectrophotometer at 490 nm. Methanol was used as blank sample before the measurements.

2.7.2.2 Succinate Dehydrogenase Activity Assay

The method of King (1967) was used for the determination of succinate dehydrogenase activity.

Reagents

0.2 M Phosphate buffer, pH 7.8

Bovine serum albumin (crystalline), in distilled water 1% (w/v)

0.6 M Succinic acid, 7.08 g in 100 ml distilled water, pH 7.8 adjusted with NaOH,

0.0015 M (2,6-dichlorophenolindophenol (DCIP) 0.043 g in 100 ml distilled water

0.009 M Phenazine methosulphate (PMS), 0.27 g in 100 ml distilled water,

0.045 M Potassium cyanide 0.293 mg in 100 ml distilled water, freshly prepared.

0.02 M sodium bicarbonate in distilled water.

STM buffer; 0.25 M sucrose, 0.1 M tricine pH 8.0 and 0.02 M magnesium sulphate (Smigielsky *et al.*, 1989).

Reaction mixture

0.75 ml phosphate buffer,

0.2 ml succinic acid

0.1 ml DCIP

0.3 ml albumin

0.2 ml phenazine methosulphate

Sterile distilled water was added to give a final volume of 2.95 ml.

Experimental Procedures

E. coli was grown on nutrient broth overnight on a orbital shaker at 30°C. The cells were harvested and washed three times with distilled water. A sterile 1 litre Erlenmeyer flask containing 500 ml filtered-autoclaved lake water was inoculated with *E. coli* to give an initial viable count of approximately 10^8 cfu/ml. 50 ml samples were centrifuged in Beckman JA 10 centrifuge (4420 g) and resuspended in 5 ml STM buffer. Further steps were carried out on ice. The samples were sonicated for 5 to 6 times one minute pulses at an amplitude of 6 until clear. The sonicated suspension was then centrifuged in Beckman JA 17 centrifuge (8820 g, 4°C). 100 μ l volume of supernatant was added to the above reaction mixture in to a cuvette of 1 cm optical path. Enzyme activity was read within 5 second after the addition of enzyme to reaction mixture at absorbance 600 nm against a water blank.

Calculations were carried out to convert observed change in absorbance mM succinate oxidised (King, 1967). The change in absorbance over each minute interval was multiplied by 0.0476 to convert the absorbance to mM succinate oxidised per minute. The extinction coefficient for the change in the oxidised states of PMS is 21, 000 (per M per cm).

2.7.2.3 NADH Dehydrogenase Activity

The NADH dehydrogenase activity was measured according to King and Howard (1967)

Solutions

2 mM N-Glycylglycine buffer, 13.2 mg in 100 ml sterile distilled water, buffered at pH 8.5 with HCl and autoclaved at 121°C for

6 mM NADH (Sigma), 213 µg in 0.1 ml of 2 mM glycylglycine buffer, pH 8.5 (prepared fresh and use in 4 h)

0.6 mM 2,6-dichlorophenolindophenol (DCIP), 17.4 mg in 100 ml sterile distilled water

Reaction mixture

To a cuvette of 1 cm optical path are added;

0.2 ml DCIP

0.35 ml glycylglycine buffer

0.1 ml of NADH

Sterile distilled water was added to give a final volume of 2.30 ml.

III- Experimental Procedures

E. coli was grown in nutrient broth overnight on a orbital shaker at 30°C. The cells were harvested and washed three times with distilled water. A sterile 1 litre Erlenmeyer flask containing 500 ml filtered-autoclaved lake water was inoculated with *E. coli* to give an initial viable count approximately 10^8 cfu/ml. 50 mls of sample was centrifuged in a Beckman J2-21 centrifuge (8820 g for 15 min) and resuspended in STM buffer (Smigielsky *et al.*, 1989). Further steps were carried out on ice. The samples were sonicated using a Jencons sonicator for 5 to 6 times one minute pulses at an amplitude of 6 until clear. After sonication the samples were centrifuged in Beckman and supernatant was used as enzyme source. Above reaction mixture was added proper volume of enzyme

and changes in activity was measured within 5 second after the addition of enzyme to the reaction mixture.

The method of (King and Howard, 1967) was used for calculation of enzyme activity. The change in absorbance over each minute interval was multiplied by 0.0476 to convert the absorbance to mM NADH oxidised per minute. The extinction coefficient for the change in the reduced states of NADH is 21,000 (per M per cm).

2.7.3 β -Galactosidase Enzyme Assay

β -galactosidase activity of *E.coli* was in lake water examined according to the method of Pardee *et al.* (1959).

Solutions

0.05 M sodium phosphate buffer, pH adjusted to 7.5

0.01 M ONPG (o-nitrophenyl- β -D-galactopyranoside) in distilled water

0.032 M Glutathione (reduced form) in sterile distilled water, stored at 4°C

1 M sodium carbonate in sterile distilled water, 0.1% w/v deoxycholate in sterile distilled water.

Experimental procedure

E.coli was grown in the basal medium overnight at 30°C. The cells were harvested and washed with 0.05 M phosphate buffer. 100 ml lake water sample were inoculated with *E.coli* to give an initial viable count of approximately 10^8 cfu/ml.

1 ml culture was removed from the flasks and added to 4 mls of 0.05 M phosphate buffer in sterile plastic universals. This mixture was added a drop of toluene and 0.1% sodium deoxycholate. 0.2 ml glutathione was added to the solution to prevent false colouration. All the samples were incubated at 30°C for 4 h. The reaction was stopped by the addition of 0.2 ml of sodium carbonate.

Total activity was expressed as change in absorbance at 420 nm/4h. Total activity was based on total mass of bacteria in the samples. However specific enzyme activity was calculated as total activity per unit of viable count.

2.8 Protein Determination Experiments

2.8.1 Determination of protein concentration

Protein estimation was carried out according to Tsukada *et al.* (1987) Bovine Serum Albumin (BSA) was used as a protein standard for establishing a standard curve.

Reagents

0.1% (w/v) BSA in distilled water

100 mg of Coomassie Brilliant Blue (G-250) was dissolved in 50 ml of 95% ethanol. Then this solution was added 100 mls of 85% (w/v) phosphoric acid and filled up to a final volume of 1 litre. This solution was light sensitive therefore it was kept in a cool dark place (4°C in the refrigerator). BSA was diluted down in 0.015 M NaCl and standard protein curve established from 100 µg to 1000 µg protein ml. 0.1 ml of sample from each aliquots was mixed with reagent dye against blank sample. Spectrophotometric measurement was performed at 595 nm.

2.8.2 Outer Membrane Protein Extraction

Outer membrane proteins were extracted according to Silhavy *et al.* (1984). *E.coli* was grown in nutrient broth or minimal medium overnight at 30°C then transferred to the filtered-autoclaved lake water under different stress and nutrient conditions. The cells were pelleted after removing them from flasks by centrifugation in Beckman J2-21 centrifuge(3440 g for 10 min). The pellet

was washed with distilled water and the supernatant was discarded. The pellet was resuspended in 5 ml ice-cold mM Tris pH 8.5, 5.2 mM EDTA and 0.5 ml of concentrated Triton x100. At the same each DNase and RNase were added to this extraction at final concentration of 100 μ g/ml. This sample was sonicated for 5 or 6 times one minute pulses at an amplitude of 6 until clear. The sonicated suspension was centrifuged in a Beckman JA 17 (13,800 g, 4°C). The pellet was discarded. The supernatant was centrifuged again in a Beckman L8 ultracentrifuge (114,562 g) for 120 min and the supernatant discarded remaining supernatant was allowed to drain by inverting the tube. Sample buffer (50 μ l) was added and the sample vortexed to resuspend the pellet before overnight storage at 4°C. The sample was warmed at 37°C, vortexed until resuspended and dispensed in 10 μ l fractions (each contains between 50-100 μ g protein per ml). These were stored at -20°C until used.

2.8.3 Cytoplasmic Proteins Extraction

The same process was followed as above but after separating outer membrane proteins by centrifugation, the supernatant was used for cytoplasmic proteins. All the samples were stored at -20°C until used. Protein concentration in the cytoplasmic sample was increased by Amicon filtration before loading the sample on the gel. Ultrafiltration using a membrane with a nominal molecular weight cut off of 10,000 daltons.

2.8.4 Whole Cell Extract

E. coli was grown in nutrient broth or minimal medium overnight at 30°C then harvested, washed and resuspended in filtered-autoclaved lake water. Sample was removed from the flasks and the cells harvested by centrifugation. The pellet was collected and resuspended in 50 mM Tris pH (8.5) containing

5.2 mM EDTA with deoxyribonuclease (100 µg/ml). The samples were sonicated as above. Centrifugation was used to remove intact cells. This sample was used as a whole cell extract and stored in 30 µl aliquots -20°C until used

2.9 Gel Electrophoresis of proteins

2.9.1 Polyacrylamide Gel Electrophoresis (PAGE)

The method employed the ionic detergent, sodium dodecyl sulphate (SDS), to dissociate proteins into their individual polypeptide subunits. A discontinuous buffer system was used with a stacking gel polymerised on top of the resolving gel (Laemmli, 1970). Just before use samples were boiled at 100°C for 5 to 10 min. Samples (30 µl) were loaded onto 11% acrylamide gel, with 3% stacking gel system and run at 60 volts on a Bio-Rad Protean gel system when the tracking dye (bromophenol blue) was through the stacking gel, the voltage was increased to 160 volts until the tracking dye had reached the bottom of the gel. Before staining the gel was placed in methanol 50% (v/v), acetic acid 10% (v/v) overnight.

Staining was carried out in 400 ml Coomassie blue (Bio Rad) (25% propan-2-ol, 10% glacial acetic acid, 0.1% Coomassie blue) overnight, followed by 2 changes of destain (25% propan-2-ol, 10% glacial acetic acid) until the background cleared.

Protein molecular weight markers (Sigma) Lysozyme 14,300, β -Lactoglobulin 18,400, Trypsinogen 24,000, Pepsin 34,700, Albumin 45,000 (ovalbumin), Albumin (bovine plasma) 66,000 were run at the same time.

The gel solutions were prepared as follows:

Tris-glycine running buffer (pH 8.3) x 10 concentration

Solution A

Tris (Trizma base) 30.3 g

Glycine 144 g

SDS 10 g

Distilled water to 1 litre, pH adjusted to 8.3 with HCl. This was used for running buffer after 10 times dilution.

Solution B

Tris (Trizma base) 30.29 g

SDS 2.0 g

Distilled water to 1 litre, pH adjusted to 6.8 with HCl. This was used as the buffer in the stacking gel.

Solution C

Tris (Trizma base) 90.86 g

SDS 2.0 g

Distilled water to 1 litre, pH adjusted to 8.8 with HCl. This was used as reservoir (running) buffer.

Solution D

Acrylamide stock

Acrylamide 30 g

NN'-Methylenebisacrylamide 0.8 g

Distilled water to 100 ml, stored in the dark at 4°C

Solution E

Ammonium persulphate solution

Ammonium persulphate 0.1 g

Distilled water to 10 ml, freshly prepared each time

Resolving gel solution

Acrylamide stock 9.9 mls

Solution C	15 ml
Distilled water	5.1 ml
Tetramethylethyl-enediamine (TEMED) 10 μ l	
Solution E	750 μ l

Stacking gel solution

Acrylamide stock	1 ml
Solution B	5 mls
Distilled water	4 mls
TEMED	8 μ l
Solution E	0.5 mls

DNase solution for membrane preparation

Tris (Trizma base) 0.606 g

EDTA 0.074 g

DNase 100 μ g

Distilled water to 100 ml, pH was adjusted to 8.5 before the addition of

DNase

Sample buffer

Solution B	25 ml
SDS	2 g
Glycerol	10 mls
2-Mercaptoethanol	5 mls
1% Bromophenol blue	0.1 ml
Distilled water to 100 mls	

Staining solutions

Isopropanol	125 ml
-------------	--------

Glacial acetic acid	50 ml
Coomassie brilliant blue	1.25 g
Distilled water	325 ml

Destain solution

Isopropanol	1 litre
Glacial acetic acid	1.4 litres
Distilled water to 20 litres	

2.9.2 Two-dimensional gel electrophoresis

This technique separates proteins according to their isoelectric point in the first dimension and according to molecular weight in the second dimension by SDS-PAGE. There are two unrelated parameters give the maximum resolution of the proteins present in complex mixture (O'Farrell, 1975). This technique is capable of resolving 1100 different proteins from *Escherichia coli* (O'Farrell, 1975). A stable pH gradient is formed using commercial ampholines and the proteins electrophoresed to equilibrium when their net charge is zero and migration ceases (O'Farrell, 1975).

The first dimension isoelectric focusing gels were carried out in 120 mm x 1.5 mm internal diameter glass tubes, washed with 0.1 M HCl for 30 min and rinsed through with distilled water. The glass tubings were sealed at the bottom with double layers of Parafilm strengthened by o-ring plastic tubes. After isoelectric focusing the gels were extruded by water pressure with a syringe and plastic tubing. After equilibration, the gel loaded onto the stacking gel of a 11% (w/v) SDS denaturing gel which was required for the 2nd. dimension.

The buffers and solutions were prepared as follows:

A Lysis buffer

9.5 M Urea (ultra pure)	5.7 g
-------------------------	-------

(w/v) Nonidet P-40	2%
(w/v) Ampholines (LKB) pH Range 5-7	1.6%
(w/v) Ampholines (LKB) pH Range 3.5-10	0.4%
Beta-mercaptoethanol	5% (v/v)

In some cases the volume of ampholines was modified as follows:

Ampholines (LKB) pH Range 5-7	0.166% (v/v)
Ampholines (LKB or Survalyt) pH Range 3.5-10	0.166% (v/v)
Ampholines Sigma) pH Range 3.5-10	0.166% (v/v)

B. Sonication buffer

0.01 M Tris-HCl buffer pH 7.4
 5 mM Magnesium chloride
 Pancreatic RNase 50 µg/ml

C. DNAase solution

Pancreatic DNase 1 mg/ml
 0.01 M Tris-HCl buffer pH 7.4
 1 mM Magnesium chloride

This buffer was stored as frozen at -20°C

D. 30% (w/v) acrylamide solution for first dimension

Acrylamide 28.38 g
 Bisacrylamide 1.62 g
 Distilled water to 100 ml

E. Nonidet P-40 solution

NP-40 10 g
 Distilled water to 100 ml

F Ammonium persulphate solution

10% (w/v) solution prepared fresh each day

G Gel overlay solution

8 M urea 4.8 g in 10 mls

This solution was stored at -20°C.

H Anode electrode solution

0.01 M phosphoric acid solution 1.12 ml concentrated acid in 2 L of water.

I Cathode electrode solution

0.02 M Sodium hydroxide solution 1.6 g per 2 L.

K Sample overlay solution

9 M urea 5.4 g per 10 mls

Ampholine pH Range 5-7 0.8% (w/v)

Ampholine pH Range 3.5-10 0.2%

This solution was stored as frozen aliquots at -20°C.

Buffers and solution for SDS-PAGE were used in second dimension as described in section in 2.10.4.1 but in some cases the percentage of acrylamide used was 44% instead of 30% and stacking gel acrylamide solution used was as 4.5% instead of 3%.

O SDS sample buffer (Laemmli sample buffer)

10% (w/v) glycerol

5% (v/v) beta-mercaptoethanol

2.3 % (w/v) sodium dodecyl sulphate (SDS) or sodium lauryl sulphate
0.0625 M Tris-HCl pH 6.8

P Agarose gel

0.1 g Agarose (Sigma type II) was melted in 10 ml of SDS sample buffer then 2 ml of 0.1 % (w/v) Bromophenol blue solution added. This solution was stored at 4°C.

Isoelectric focusing gel mixture

Urea	5.5 g (w/v)
30 % (w/v) Acrylamide stock	1.33 ml
10 % (w/v) Nonidet P-40	2.0 ml
Ampholine pH Range 5-7.0	0.4 ml
Ampholine pH Range 3.5-10	0.1 ml
Distilled water to 10 ml	
TEMED	7 μ l
10 % Ammonium persulphate	10 μ l

Isoelectric gel mixture was vortexed under vacuum then 10 μ l 10 % (w/v) ammonium persulphate and 7 μ l TEMED were added immediately before filling the glass tubes. The glass tubes were filled with the gel mixture using a 1 ml glass pipette avoiding the introduction of air bubbles into the tubes. This was carried out in a 30°C warm room to avoid urea crystallisation in the tubes. The gel was overlayed with a solution of 8 M urea (solution K) and allowed to set for 1-2 hours. The overlay solution was removed and replaced with 20 μ l of Lysis buffer (solution A) and itself overlayed with distilled water. After 2 hours, the water and buffer were poured away, 20 μ l of fresh lysis buffer (solution A) added and the tube filled with 0.02 M sodium hydroxide. Dialysis tubing was replaced with parafilm at bottom of the glass tube and strengthened with rubber

'O' rings in order not to have any air bubbles. The upper gel tank with tubes was mounted in the electrophoresis tank. The lower chamber was filled with the anode electrolyte, 0.01 M phosphoric acid (solution H) and upper tank filled with the cathode electrolyte, 0.02 M sodium hydroxide (solution I). The gels were pre-run at 200 V for 15 min, 300 V for 30 min and 400 V for 30 min. After removing lysis buffer and sodium hydroxide solution from the tubes, the samples were loaded, overlayed with 10 μ l of Overlay solution and itself overlayed with 0.02 M sodium hydroxide solution. The 0.02 M sodium hydroxide solution was poured away from upper tank and then refilled and the protein electrofocused at 400 V for 16 h and finally at 800 V for an extra one hour. The gels were extruded from the tubes by a syringe with a water pressure into 5 ml of SDS sample buffer at room temperature for 1-2 hours. The gels were immediately loaded onto the second dimension gel or stored in sample buffer at -20°C.

The second dimension gel was used as the method given in SDS-PAGE protein electrophoresis (section 2.9.1). The only differences that stacking gel was filled up to produce a space of about 3 mm from the top of the glass plates and made a well for marker dye. The isoelectric focusing gel was placed on a piece of aluminium foil. 1% agarose solution was made ready and well of the glass plates were filled with this solution and first dimension gel applied carefully without having any air bubbles at this stage. At the same time marker protein was also loaded and upper reservoir of gel rig (Bio-Rad Protean gel system) was transferred to the lower reservoir filled up with running buffer. Electrophoresis in the second dimension was carried out at 15 mA for about 16 h at room temperature.

2.9.5 Silver staining for two dimensional gel electrophoresis

All the gels were stained according to Wray *et al.* (1981). Briefly, the method was used as follows:

- 1) The gels were soaked in two changes of 50% (v/v) methanol.
- 2) 1.6 g of silver nitrate was dissolved in 8 ml of distilled water.
- 3) 42 ml 0.36% sodium hydroxide 2.5 ml ammonia solution were mixed.
- 4) 8 ml of silver nitrate solution was added to the above solution dropwise until solution cleared, then total volume made up to 200 ml with distilled water.
- 5) Gels were stained for 15 minutes.
- 6) Gels were washed twice with distilled water for 5 minutes.
- 7) A developer solution was prepared which 2.5 ml 1% (w/v) citric acid, and 0.4 ml of formaldehyde, made up to 500 ml distilled water and the gels placed in this developer solution until bands or spots appeared about 15 mins).
- 8) The developer solution was now removed and gels were placed in a solution stain stop solution of 225 ml methanol 50 ml acetic acid, made up to 500 ml in distilled water to stop staining were stored in this solution.

When destaining was necessary this was carried out before the stain stop solution was added. The destain was 50% methanol and 30% acetic acid in distilled water.

Chapter III. The effect of different stress conditions on the survival of *Escherichia coli* lake water.

3.1 Introduction

3.1.1 Starvation stress

Survival may be defined as the maintenance of bacterial genome under adverse conditions. Many studies have now been carried out on the survival of bacteria under nutrient-limited conditions especially using oligotrophic marine bacteria which have to be capable of surviving under extreme energy and nutrient starvation. Starvation-induced morphological and physiological changes have been studied in detail for several marine bacterial species (Morita, 1982; Baker *et al.*, 1983; Marden *et al.*, 1985; Hood *et al.*, 1986; Jouper-Jaan *et al.*, 1986; Nyström *et al.*, 1986).

Many bacteria produce specialised survival forms such as the endospore of *Bacillus* spp., the exospores of *Streptomyces* spp. and the cysts of *Azotobacter* spp. to enable them to survive almost indefinitely under nutrient-limited conditions. On the other hand, non-spore forming bacteria can also survive for considerable periods of time under conditions of nutrient depletion of an endogenous energy supply by reducing their cell size and metabolic activity (Morita, 1982). Some bacterial species respond to starvation by undergoing miniaturisation, a process of reductive division by which the cells continue to divide without first increasing their biomass hence the cells become progressively smaller (Morita, 1985). The cell-size reduction is one of the steps which leads to the formation of the viable but non-culturable state under starvation stress. When indicator bacteria such as *E.coli* enter this state their detection becomes a problem and of concern for public health (Novitsky and Morita, 1977; Dawson *et al.*, 1981; Colwell *et al.*, 1985). For instance Roszak and Colwell (1987) have shown that *E.coli* and *S. enteritidis* stressed in an aquatic ecosystem become non-culturable and could not be detected on standard selection media although the cells were still determined to be viable by direct count techniques.

Temperature as well as starvation can also be a factor in determining the entry of a cell into a viable but non-culturable state (Oliver *et al.*, 1991; Wolf and Oliver, 1992). The suggested that *V. vulnificus* enters a dormant state when the cells are subjected to temperature below 10°C. The bacterial cells appeared to respond to the decreasing temperature by immediately decreasing their culturability. Again this has implications for the isolation of mesophilic bacteria such as indicator strains from cold rivers and lakes. Nilsson *et al.* (1991) suggested that it may be possible resuscitate viable but non-culturable cells of *V. vulnificus* without the addition of exogenous nutrients simply with a gradual increase in temperature over a 2 to 3 day period. The occurrence of viable but non-culturable forms of indicator bacteria in aquatic ecosystems will pose a threat to public health unless because the numbers of indicator organisms will be significantly underestimated on normal selective media (Colwell, 1983).

Survival studies have for the last decade concentrated on the physiology of bacteria under starvation conditions. *E. coli* can produce at least 66 new proteins when subjected to nutrient deprivation (Nystrom *et al.*, 1989). Some of these proteins are necessary for long-term survival. Some are part of the general stress response of the bacteria to any shock, others are specific for starvation stress. Other changes which occur include, changes to the cellular concentration of RNA, and DNA, a decrease in ribosome numbers, the production of new enzymes and proteins, a lower respiration rate and a reduction in the concentration of intracellular energy and reserve materials.

3.1.2 Osmotic stress

The ability of enteric bacteria to adapt to fluctuation in the ambient osmolality is of fundamental importance for their survival if they are released into the marine environment for instance from a sea-outfall. Bacterial cells must be capable of maintaining their cytoplasmic osmotic pressure at a higher level than that of the environment (Munro *et al.*, 1989). If *E. coli* are exposed to high

saline conditions i.e. they are osmotically shocked, they respond by regulating the osmotic pressure inside the cells in order to maintain the optimum pressure for growth and enzyme activity (Booth *et al.*, 1988). *E.coli* cells accumulate chemicals which can act as osmoprotectants. This involves the synthesis of a group of proteins in response to osmotic stress (Clark and Parker, 1984). The osmolarity of the medium strikingly affects the proportionate amount of two proteins, OmpC and OmpF. The former is preferentially expressed in cells grown in a medium with a high osmolarity whilst the latter requires a low osmolarity medium for expression. This regulation can be altered by the addition of glycinebetaine to the medium (Barron *et al.*, 1986). Glycinebetaine is an osmoprotectant which can be used to rescue cell from the effects of osmotically induced stress. It can be used to protect cells from decay in seawater (Gauthier *et al.*, 1987). Bacteria can accumulate amino acids for example proline (Britten and McClure, 1962) and glutamate (Measures, 1975) as well as glycinebetaine (LeRudulier and Bouillard, 1983; Smith *et al.*, 1988) in response to osmotic stress. The synthesis of these compounds is also induced by osmotic shock. LeRudulier and Bouillard (1983) also showed that although proline, glutamate and glycinebetaine were not used by these bacteria as carbon and nitrogen sources growth and maintenance of viability were enhanced in their presence in cells subjected to osmotic shock.

3.1.3 Chlorination stress.

Chlorination is the final step in the water purification process and is used to kill any microorganisms to ensure the safety of drinking water supplies (Pelczar *et al.*, 1981). Recently many researchers shown have that chlorination does not kill all the organisms in water. Some are only sublethally injured and can recover without losing their pathogenic potential (Camper and McFeters, 1979; Singh *et al.*, 1986; Grimes and Colwell, 1986). Coliforms are as susceptible to chlorination stress as any other bacterial species (LeChevallier *et*

al., 1985) Chlorination is a non-specific oxidative agent which can oxidise proteins and enzymes, thus inhibiting the organisms metabolic capability and react with nucleotides, thus preventing reproduction (Camper and McFeters, 1979). More than 90% of an *E.coli* population exposed to low levels of chlorination showed evidence of sublethal injury or stressed but they remained viable (although often non-culturable) until conditions were suitable for their recovery (Camper and McFeters, 1979). As there are a number of stress proteins synthesised in response to chlorination shock some of these may be related to the proteins produced under starvation stress. It is known for example that starvation reduces the susceptibility of bacteria to the effects of chlorine.

3.1.4 Other stresses

Sublethal concentrations of hydrogen peroxide also result in metabolic changes in *E.coli* (Farr and Kogoma, 1991). All organisms which use molecular oxygen have to defend themselves against the toxic effects of hydrogen peroxide produced as a side product of oxygen metabolism (Fridovich, 1978). Hydrogen peroxide is toxic to many types of cell (Demple and Halbrook, 1983) causing oxidative damage to DNA and proteins (Booth *et al.*, 1988; Farr and Kogoma, 1991). Sublethal concentrations of hydrogen peroxide cause the induction of catalase and, possibly, superoxide dismutase (the enzymes involved in the degradation of hydrogen peroxide (Finn and Condon, 1975; Fridovich, 1976; Richter and Coewen, 1981) as well as of at least 30 other proteins (Farr and Kogoma, 1991). Some of these proteins are involved in the global response to stress as *E.coli* and *S. typhimurium* exposed to sublethal concentrations of hydrogen peroxide show enhanced resistance to heat and chlorination (Jenkins *et al.*, 1988). It is therefore possible that oxidative challenge could influence the survival of these organisms under starvation stress.

Exposure of *E.coli* to sublethal concentrations of the detergent, sodium dodecyl sulphate (SDS) also leads to the synthesis of novel proteins (Adamowicz

et al., 1991). At least 4 proteins are switched on and 15 others show elevated synthesis. At the same time at least 13 proteins are decreased in concentration.

Sublethal concentrations of ethanol have a similar effect with new proteins being synthesised in *E.coli* (Singleton and Sainsbury, 1987). Again it is possible that exposure of *E.coli* to sublethal concentrations of these substances could cause enhanced resistance to the effects of starvation.

3.1.5 The effects of antibiotics on bacterial survival

The term 'antibiotic' refers to chemical substances, natural or man-made which at low concentrations inhibit the growth of microorganisms (Russell and Queanel, 1983). In the natural environment antibiotics can play an important role in the competitive relationship between aquatic microorganisms and hence they have a role to play in the survival of enteric bacteria. The functions in a bacterial cell which may be affected by antibiotics include cell wall synthesis, protein synthesis and DNA synthesis. It is unlikely that *E.coli* encounters antibiotics in the natural environment but experiments have been carried out here to investigate the effects of selection of antibiotics on starved *E.coli*. The addition of antibiotics could have two effects:

1. against *E.coli* which would demonstrate that physiologically they are capable of taking up the antibiotic and are still metabolically functional;
2. against the natural microflora in which case *E.coli* would be able to survive better as any competitors would be eliminated.

Changes in the outer membrane of Gram-negative bacteria including *E.coli* can affect the sensitivity of the bacteria to antibiotics (Osborn *et al.*, 1974). Resistance is not necessarily conferred through the production of an enzyme for the destruction of the antibiotic. Hence it might be expected that changes to the outer membrane of Gram-negative bacteria conferred through the response to stress would also affect the sensitivity of the bacteria towards antibiotics. An antibiotic such as nalidixic acid, an inhibitor of DNA synthesis,

which exerts an effect in growing cells but not on non-growing cells would also be ineffective if bacteria cannot replicate their genome under starvation stress (Goss *et al.*, 1965). It might equally be expected that inhibitors of protein synthesis, such as kanamycin, streptomycin and spectinomycin, would reduce the ability of bacteria to respond to starvation or other stresses if the response requires the *de novo* synthesis of stress proteins.

3.2 Results

3.2.1 The effect of starvation stress on *Escherichia coli* survival in lake water

Starvation stress is one of the important factors which affects the survival of bacteria in aquatic environments. The survival of *E. coli* in the absence and presence of the natural microflora in filtered-autoclaved and natural lake water samples at different temperatures was compared. Figure 3.1 and Figure 3.2 show that the die off or disappearance of *E. coli* under starvation stress is inversely related to the incubation temperature. The viability of *E. coli* was reduced at a faster rate at 37°C than at other incubation temperatures. Flint (1987) and Rhodes and Kator (1988) have also reported that temperature played an important role in the survival of *E. coli* in freshwater environments. In untreated lake water survival was longer at 4°C than at 15°C or 37°C probably due to differences in metabolic activities of the cells and/or the effectiveness of protozoan predation at these different temperatures. However there was a little difference between survival of *E. coli* at temperatures between 4°C and 30°C in filtered-autoclaved lake water for up to 20 days of incubation. Over this period the population size showed no significant variation. Repeated experiments showed that there was always a dramatic loss of viability in *E. coli* incubated at 37°C in sterile and natural water. At this temperature either *E. coli* are rapidly entering a viable but non-culturable state or are genuinely becoming non-viable cells. The temperature dependence of the formation of the viable but non-culturable state has previously shown for *Vibrio vulnificus* (Wolf and Oliver, 1992).

There was a little difference between the survival of cells grown in phosphate-limited, nitrogen-limited minimal medium, complete minimal medium or nutrient broth in filtered-autoclaved lake water (Fig. 3.3) or untreated lake water (Fig. 3.4). In filtered-autoclaved lake water the cells survived for at least

180 days at 15°C with no decline in viability. There was a slight increase in the viable count of the cells grown in the minimal medium and nitrogen-limited medium over the incubation period but this was not significant. In the untreated lake water there was a little difference in the rate of decline for the cells grown in any of the minimal media. The only visible difference was the complete disappearance of cells grown in nutrient broth between day 4 and 5 whereas a residual population of cells grown in minimal medium was always left.

3.2.2 Morphological changes of *Escherichia coli* under long-term starvation stress

Morphological changes of *E. coli* were observed under starvation stress at different temperatures by means of electron microscopy, epifluorescence microscopy and the Coulter counter. Epifluorescence microscopy of cells stained with acridine orange showed that there was a reduction in the cell size of *E. coli* under starvation stress in lake water (Fig. 3.5). After 96 days at 15°C acridine orange staining revealed that most *E. coli* were nearly spherical in shape and assumed to be viable. An increase in the cells which were apparently non-viable (green cells in Fig. 3.5b) was also noted. This observations was also supported by electron microscopy examination of starved cells. These became more irregular shaped after 20 days of starvation at 37°C (Fig. 3.6). Similar morphological changes were reported for *E. coli* by Reeve *et al.* (1984) who showed that the cell cytoplasmic contents shrunk away from the cell membrane leading to 'crumpled' looking cells. However, some cells were able to maintain their shape throughout the starvation period. The relationship between these various morphological changes and the viable but non-culturable state was not known.

The effect of temperature on the reduction of cell size was also followed using the Coulter counter to measure cell volume changes. Figure 3.7 shows

that although *E.coli* maintained their original cell size at 4°C, a proportional decrease in cell volume occurred in response to an increase in temperature in filtered-autoclaved lake water under starvation stress. As a consequence the largest cell size reduction took place at 37°C. It was also noted that the most severe loss of viability was detected in *E.coli* cells under starvation conditions at 37°C. In conclusion a strong positive relationship existed between the reduction in cell size, temperature and starvation survival in filtered-autoclaved lake water.

3.2.3 The effect of starvation on the detection of *Escherichia coli* in lake water

It has been suggested that if cells become viable but non-culturable then plate counts will become an unsatisfactory method for the estimation of the total number of viable bacteria present in environmental samples. Here the number of *E.coli* in filtered-autoclaved lake water was determined. Figure 4.8 shows that there was an increase in the total direct count over a 20 day period at 15°C and 25°C but the number of respiring cells in the sample and the viable plate count remained constant or fell slightly. At 4°C there was no increase in total count but it again was higher than the respiring cell count and the plate count over the incubation period. After 30 day incubation there was a large difference between the plate count, direct count and the count of respiring cells. This was assumed to be due to the influence of viable but non-culturable cells on the counts. As shown in Figure 3.8b approximately 90% of *E.coli* population remained metabolically active but non-culturable in filtered-autoclaved lake water under starvation stress. If one assumes that if the ability to respire is a measure of viability then between 5% and 10% of starved cells are counted by the plate count method even using a non-selective agar. If a selective agar had been

Figure 3.1 Survival of *Escherichia coli* in untreated lake water.

Figure 3.2 Survival of *Escherichia coli* in filtered autoclaved lake water

E. coli was grown in nutrient broth overnight at 30°C, harvested, washed and resuspended in sterile lake water. 250 ml microcosms containing 100 ml lake water were inoculated to give an initial viable count of approximately 10⁶ cfu/ml. Flasks were incubated in the dark without shaking at different temperatures. Viable counts were determined by surface spread plates on nutrient agar plates after incubation at 37°C for 24 h.

Fig. 3.1

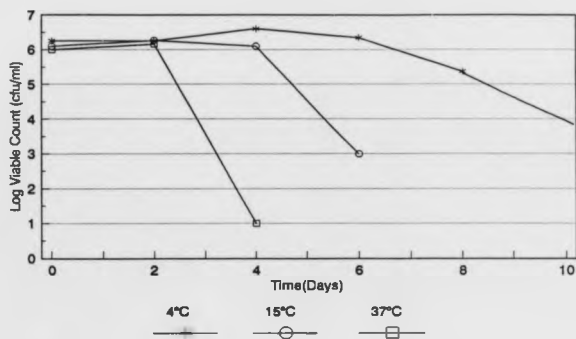


Fig. 3.2

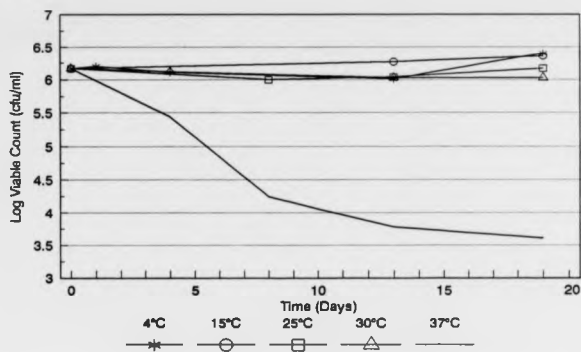


Figure 3.3 The influence of growth medium on the survival of *Escherichia coli* in filtered autoclaved lake water

Figure 3.4 The influence of growth medium on the survival of *Escherichia coli* in untreated lake water

E. coli was grown in nutrient broth, minimal medium, phosphate-limited or nitrogen-limited minimal medium overnight at 30°C, harvested, washed and resuspended in sterile lake water. 250 ml microcosms containing 100 ml lake water were inoculated to give an initial viable count of approximately 10^6 cfu/ml. Flasks were incubated in the dark without shaking at 15°C. Viable counts were determined by surface spread plates on nutrient agar plates after incubation at 37°C for 24 h.

Fig. 3.3

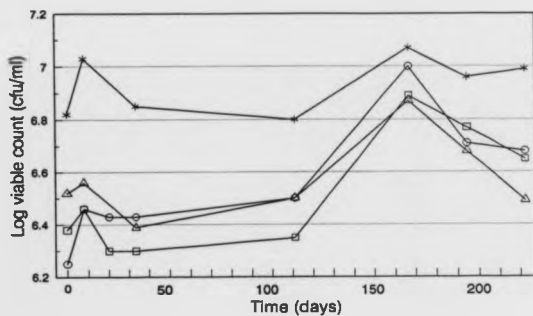


Fig. 3.4

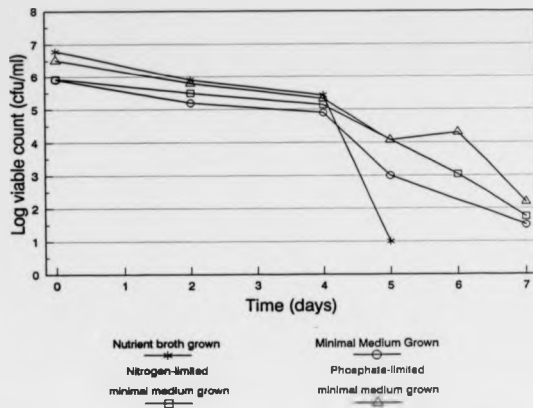


Figure 3.5 The morphological changes of *E.coli* under starvation stress in filtered-autoclaved lake water by epifluorescence microscopy

E.coli was grown in nutrient broth overnight at 30°C , harvested, washed and resuspended in sterile lake water. 250 ml microcosms containing 100 ml lake water were inoculated to give an initial viable count of approximately 10^6 cfu/ml. Flask were incubated in the dark without shaking at 15°C. Cells were examined at day 0 (Fig. 3.5a) and after 96 days of starvation (Fig. 3.5b). Cells were examined under epifluorescence microscopy using acridine orange as a stain.

Figure 3.5a *Escherichia coli* cells at day 0



Figure 3.5b *Escherichia coli* cells after 96 days of starvation in filtered-autoclaved lake water

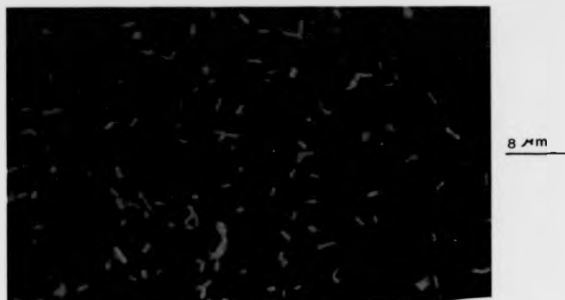


Figure 3.6 The morphology of *Escherichia coli* in filtered-autoclaved-lake water by electron microscopy

E. coli was grown in nutrient broth overnight at 30°C, harvested, washed and resuspended in sterile lake water. 250 ml microcosms containing 100 ml lake water were inoculated to give an initial viable count of approximately 10^6 cfu/ml. The flask were incubated in the dark without shaking at 30°C. Samples were examined under the transmission electron microscopy using phosphotungstic acid as a negative stain after 0 day (Fig. 3.6a), 20 days (Fig. 3.6b) and 30 days (Fig. 3.6c).

Figure 3.6a *Escherichia coli* at day 0

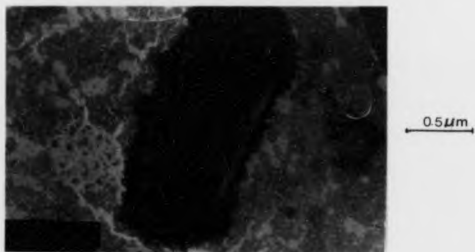


Figure 3.6b *Escherichia coli* cells after 20 days of starvation.



Figure 3.6c *Escherichia coli* cells after 30 days of starvation.



Figure 3.7 Cell size changes of *Escherichia coli* in filtered-autoclaved lake water

E. coli was grown in nutrient broth overnight at 30°C, harvested, washed and resuspended in sterile lake water. 250 ml microcosms containing 100 ml lake water were inoculated to give an initial viable count of approximately 10^6 cfu/ml. The flask were incubated in the dark without shaking at different temperatures. cell size changes measured at different intervals by the Coulter counter. The Coulter counter records the size of the majority of the sample.

Fig.3.7

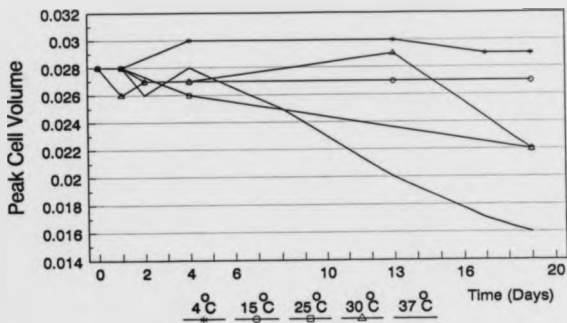


Figure 3.8 Comparison of the viable count, total direct count and respiring cell count of *Escherichia coli* starved in filtered-autoclaved lake water

E. coli was grown in nutrient broth overnight at 30°C , harvested, washed and resuspended in sterile lake water. 250 ml microcosms containing 100 ml lake water were inoculated to give an initial viable count of approximately 10^6 cfu/ml. The flask were incubated in the dark without shaking at 4°C (Fig. 3.8a), 15°C (Fig. 3.8b) or 25°C (Fig. 3.8c). Viable counts (VC) were determined by surface spread plate technique on nutrient agar plates after incubation at 37°C for 24 h. Total direct counts (TDC) were obtained by epifluorescence microscopy after acridine orange staining. Respiring cell count (RCC) was obtained by epifluorescence microscopy of acridine-orange staining stained cells incubated with INT-violet.

Fig. 3.8a

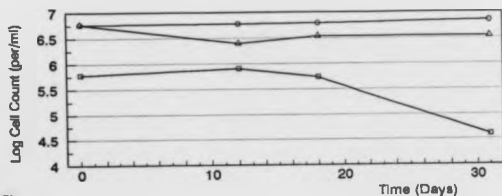


Fig. 3.8b

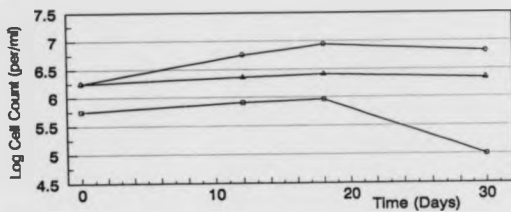
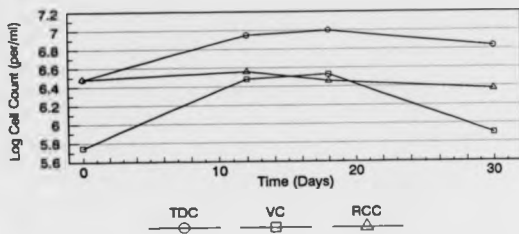


Fig. 3.8c



included the plate count would have been lower (Flint, personal communication). The results suggest that *E.coli* does enter a viable but non-culturable state under starvation stress in filtered-autoclaved lake water.

3.2.4 The effect of osmolarity and osmoprotectants on the survival of *Escherichia coli* in lake water

Osmolarity is another of the factors which can affect the survival of *E.coli* in aquatic, especially marine, environments. Bacteria subjected to osmotic shock can synthesise or accumulate osmoprotectants, such as glycinebetaine or amino acids, in order to effect on cell viability. In this experiment glutamate, proline and glycinebetaine were used as osmoprotectants for *E.coli* subjected to osmotic shock in filtered-autoclaved lake water amended with 5% NaCl. Figure 3.9 shows that *E.coli* gradually lost viability over the first 10 days but the addition of the osmoprotectants after this period led to a recovery in the viable count. This was most notable with proline (an increase of 4.9 times) than with glutamate (2 times) and glycinebetaine (2.2 times). These results suggest that *E.coli* becomes stressed after the exposure to osmotic shock and enters a viable but non-culturable state. It can be recovered from this state after the addition of osmoprotectants.

3.2.5 The effect of chlorination on the survival of *Escherichia coli* in lake water

Chlorination is used as the final step of water treatment to ensure that water entering the distribution system is effectively safe to drink. Here the effects of chlorination on *E.coli* subjected to starvation conditions in untreated (Fig. 3.10c) and filtered-autoclaved (Fig. 3.10a) lake water were studied. Chlorine was added in the form of sodium hypochlorite (the active form of chlorine in water). Increasing concentrations of sodium hypochlorite resulted in a greater decline in the viable count of *E.coli* although the effect was more

pronounced in untreated lake water where *E. coli* would be competing for the nutrients necessary for it to survive this additional stress. Although the t_{90} values do not differ significantly between the untreated and filtered-autoclaved lake water amended with 145 mM (t_{90} 1.5 and 1.5 days respectively) or 290 mM sodium hypochlorite (t_{90} 1.2 and 1.5 days respectively). The residual population in both cases is higher in the filtered-autoclaved lake water samples and also higher with the lower concentration of hypochlorite. The total direct counts (Fig. 3.10b) showed no significant decline in numbers over the incubation period although the plate count had declined by at least 3 logs in the case of 290 mM hypochlorite amendment. Indeed there was some evidence that the population size increased with the two highest concentrations of hypochlorite. This could be a response to stress similar to the reductive division seen with starvation stress in marine vibrios. This increase was not matched with an increase in the viable plate count. Although no data for the numbers of respiring cells were obtained in this part of the experiment it is suggested that the difference between the viable counts and the direct counts could once again be a reflection of the formation of a viable but non-culturable form in response to stress imposed by starvation and by chlorination.

3.2.6 The effect of pretreatment with hydrogen peroxide, sodium dodecyl sulphate and ethanol on the survival of *Escherichia coli* in lake water.

Sublethal concentrations hydrogen peroxide, sodium dodecyl sulphate (SDS) and ethanol result in metabolic changes in *E. coli* cells. There have been some suggestions that exposure to sublethal concentration of oxidising agents can lead to an increase in resistance to other forms of stress. Therefore the relationship between survival under starvation conditions and pretreatment with hydrogen peroxide, SDS and ethanol was investigated. *E. coli* was grown in nutrient broth supplemented with the above chemicals then transferred to filtered-autoclaved lake water (Fig. 3.11) or untreated lake water (Fig. 3.12)

Figure 3.9 The effect of osmolarity and osmoprotectants on the survival of *Escherichia coli* in filtered-autoclaved lake water.

E. coli was grown in nutrient broth overnight at 30°C, harvested, washed and resuspended in sterile lake water. 250 ml microcosms containing 100 ml lake water were inoculated to give an initial viable count of approximately 10^6 cfu/ml. The lake water was amended with 5% NaCl. The flask were incubated in the dark without shaking at 15°C. Viable counts (VC) were determined by surface spread plate technique on nutrient agar plates after incubation at 37°C for 24 h. After 10 days incubation the flasks were aseptically amended with proline (final concentration 0.1 M), glycinebetaine (final concentration 0.25 M) or glutamate (final concentration 0.2 M).

Fig. 3.9

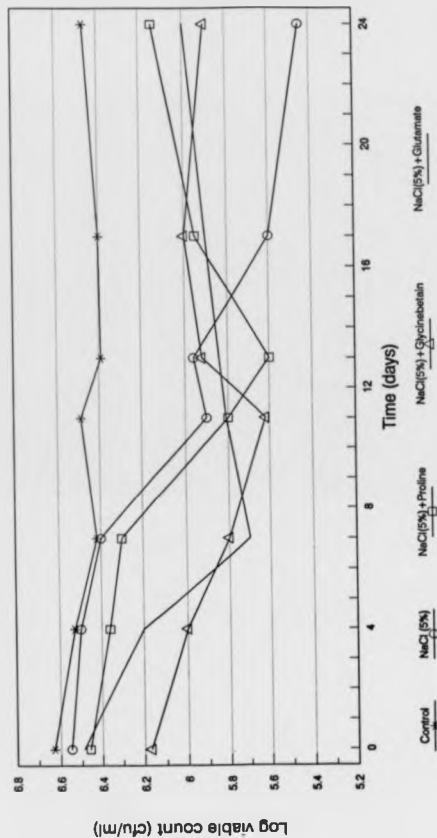


Figure 3.10 The effect of chlorine stress on the survival of *Escherichia coli* in lake water.

E. coli was grown in nutrient broth overnight at 30°C , harvested, washed and resuspended in sterile lake water. 250 ml microcosms containing 100 ml lake water (Fig. 3.10a and Fig. 3.10b) or untreated lake water (Fig. 3.10c) were inoculated to give an initial viable count of approximately 10^6 cfu/ml. The flask were incubated in the dark without shaking at 15°C. Viable counts were determined by surface spread plates on nutrient agar plates after incubation at 37°C for 24 h. Total direct count (TDC) were determined by epifluorescence microscopy of acridine-orange stained samples.

Fig.3.10a

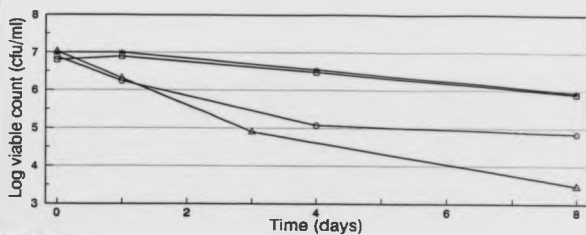


Fig.3.10b

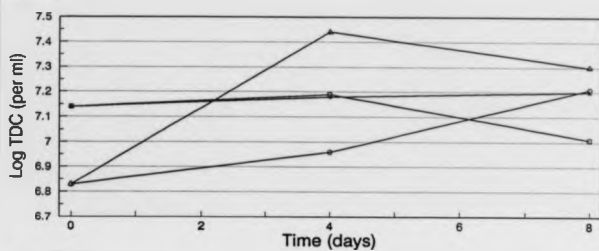
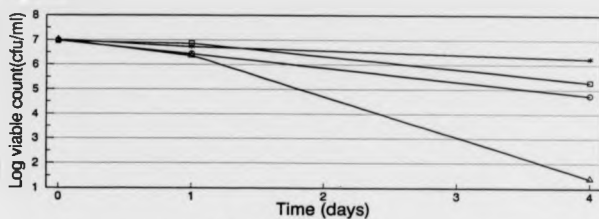


Fig.3.10c



control Sodium hypochlorite

—●— .029 M .145 M .29 M

—■— —○— —▲—

and incubated at 15°C. In filtered-autoclaved lake water cells pretreated with SDS and hydrogen peroxide lost viability over the first 5 days of incubation. The population then showed a significant increase before stabilising at a level 0.5 log below that of the untreated control after 27 day. Ethanol-pretreated cells increased rapidly in numbers over the first 2 days before maintaining a population size the same as the control. The untreated control numbers remained unchanged over the 27 days incubation period. These results suggest that *E.coli* could overcome the effects of ethanol pretreatment upon starvation but not the injury caused by pretreatment with hydrogen peroxide and SDS.

Pretreated *E.coli* were also inoculated into untreated lake water and incubated at 15°C. Figure 3.12 shows that the pretreatments did not affect the survival of *E.coli* compared to untreated control. The tgg values were the same for all treatments (2 to 2.3 days). No growth was seen in these samples unlike with the filtered-autoclaved lake water samples. It was concluded that pretreatment did not affect the survival of *E.coli* in untreated lake water and that the cells were unable to repair damage due to their inability to compete with the natural microflora for the nutrients necessary to effect repair.

The effect of oxidation stress was also determined for nutrient broth-grown *E.coli* transferred to filtered-autoclaved lake amended with hydrogen peroxide at final concentrations from 2.64 mM to 8.8 mM and incubated at 15°C. Figure 3.13 shows that only the highest two concentration caused a decline in the *E.coli* numbers. The continued presence of an agent capable of causing oxidative stress does have an effect on the survival of *E.coli* whereas pretreatment with the agent has only limited effect. No respiring cell counts were determined here therefore no comment can be made on the formation for a viable but non-culturable form in response to this dual stress.

3.2.7 The effect of antibiotics on the survival of *Escherichia coli* under starvation stress

Antibiotics are only effective as antimicrobial agents against bacteria which are growing or metabolically active. The aim of this set of experiments was to determine the effects of antibiotics on the survival of *E. coli* in lake water under starvation stress in the presence and absence of the natural microbial flora at different temperatures. Most of the antibiotics used were inhibitors of different stages of protein synthesis.

The effect of spectinomycin on the survival of *E. coli* is shown in Figure 3.14a, Table 3.1 and Table 3.2. In untreated lake water (Fig. 3.14a) spectinomycin had little effect at 4°C or 15°C but extended the survival of *E. coli* by a significant period at 37°C. At this temperature spectinomycin may be having a greater effect on the natural microflora thus allowing *E. coli* to compete more efficiently for nutrients than on *E. coli* itself. In filtered-autoclaved lake water there was no significant differences between t_{90} values at any of the incubation temperatures. Using different concentrations of spectinomycin showed that only a final concentration of 50 $\mu\text{g/ml}$ effectively reduced the t_{90} value in both filtered-autoclaved and untreated lake water at 37°C.

The effects of the addition of streptomycin to the lake water are shown in Figure 3.1 and Table 3.3. In untreated lake water the addition of a final concentration of streptomycin of 0.5 $\mu\text{g/ml}$ did not alter the t_{90} value in untreated lake water. Again only the largest concentration tested had any effect on the t_{90} value in untreated lake water (Table 3.3) whereas in filtered-autoclaved lake water all the concentrations reduced the t_{90} value with the largest decrease again being associated with the highest concentration of streptomycin.

The effect of the addition of neomycin are shown on Figure 3.14c and Table 3.1. In untreated lake water the addition of neomycin again had an effect on the t_{90} value at 15°C and 37°C. In both instances the t_{90} value was higher in

Figure 3.11 The effect of hydrogen peroxide, sodium dodecyl sulphate and ethanol pretreatment on the survival of *Escherichia coli*.

E. coli was grown in nutrient broth with and without the addition of 18 mM hydrogen peroxide, 0.5% w/v sodium dodecyl sulphate or 1% w/v ethanol overnight at 30°C, harvested, washed and resuspended in sterile lake water. 250 ml microcosms containing 100 ml filtered-autoclaved lake water were inoculated to give an initial viable count of approximately 10^7 cfu/ml. The flask were incubated in the dark without shaking at 15°C. Viable counts were determined by surface spread plates on nutrient agar plates after incubation at 37°C for 24 h.

Fig. 3.11

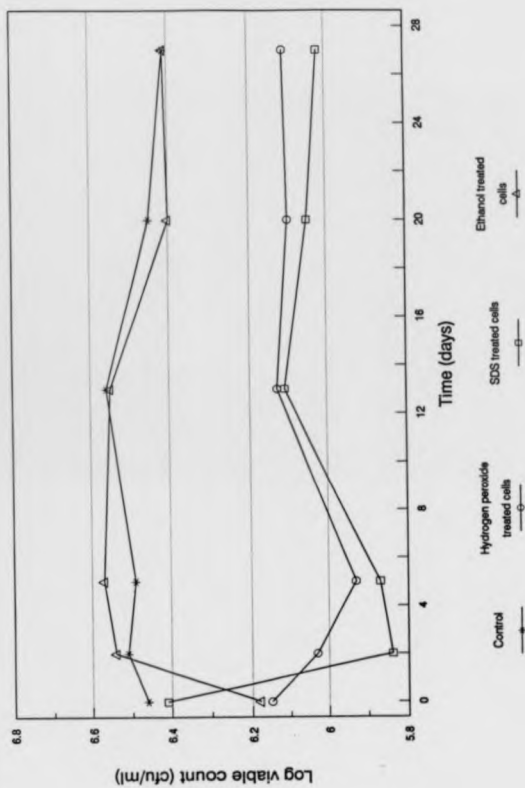


Figure 3.12 The effect of hydrogen peroxide, sodium dodecyl sulphate and ethanol pretreatment on the survival of *Escherichia coli*.

E. coli was grown in nutrient broth with and without the addition of 18 mM hydrogen peroxide, 0.5% sodium dodecyl sulphate or 1% ethanol overnight at 30°C, harvested, washed and resuspended in sterile lake water. 250 ml microcosms containing 100 ml untreated lake water were inoculated to give an initial viable count of approximately 10^7 cfu/ml. The flask were incubated in the dark without shaking at 15°C. Viable counts were determined by surface spread plates on nutrient agar plates after incubation at 37°C for 24 h.

Figure 3.13 The effect of hydrogen peroxide on the survival of *Escherichia coli*

E. coli was grown in nutrient broth overnight at 30°C, harvested, washed and resuspended in sterile lake water. 250 ml microcosms containing 100 ml filtered-autoclaved lake water amended with hydrogen peroxide at final concentrations between 2.64 mM to 8.8 mM were inoculated to give an initial viable count of approximately 10^7 cfu/ml. The flask were incubated in the dark without shaking at 15°C. Viable counts were determined by surface spread plates on nutrient agar plates after incubation at 37°C for 24 h.

Fig.3.12

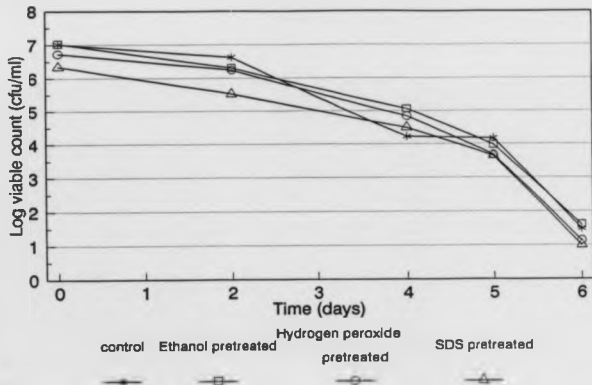
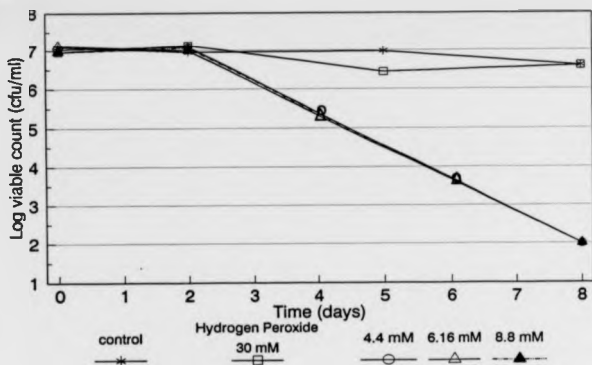


Fig.3.13



the amended flask than in the unamended flask. In filtered-autoclaved lake water the addition of neomycin reduced the t_{90} value suggesting that although neomycin may have an effect on *E. coli* in lake water in the presence of the natural microflora more the neomycin is active against the natural microflora than against *E. coli*.

The effects of increasing concentrations of ampicillin at 37°C and 15°C are shown in Table 3.4. In both filtered-autoclaved and untreated lake water only 100 µg/ml had any effect on the t_{90} value at 15°C and 37°C. This was a surprising result as the minimum inhibitory concentration (MIC) for this antibiotic in nutrient broth is less than 5 µg/ml (Flint, personal communication).

3.2.7.1 The effects of addition of ammonium sulphate and antibiotics on the survival of *Escherichia coli* in lake water.

In chapter 4 it was shown that ammonium sulphate enhanced the survival of *E. coli* in untreated lake water. Here the effects of simultaneously exposing cells to ammonium sulphate and nalidixic acid (Fig. 3.15) or kanamycin (Fig. 3.16) in lake water was examined.

Nalidixic acid is an inhibitor of DNA replication in bacteria. Figure 15 shows that *E. coli* numbers declined as fast in the presence of nalidixic as in its absence. This suggested that DNA synthesis was not taken place under these starvation conditions (at 15°C in untreated lake water). When ammonium sulphate was also added to the lake water, survival in the presence and absence of nalidixic acid was enhanced to the same degree. The only difference between survival in the presence and absence of ammonium sulphate was that when no nalidixic acid was present the bacterial population increased over the first days of starvation. When nalidixic acid was also present there was no increase but equally no dramatic decline in numbers. If DNA synthesis was taking place in the presence of ammonium sulphate leading to an increase in number then one would have expected that, with nalidixic acid present, numbers would have

declined. Nalidixic acid was used here at 100 $\mu\text{g/ml}$, over ten times the MIC for this antibiotic in nutrient broth (Flint, personal communication).

Kanamycin is an inhibitor of protein synthesis in bacteria. The MIC for this organism was less than 2 $\mu\text{g/ml}$ (Flint, personal communication). Here it was used at final concentrations of 10 and 100 $\mu\text{g/ml}$ in filtered-autoclaved lake water. Figure 3.16 shows that kanamycin had a dramatic effect on *E.coli*. The addition of both concentrations decreased the t_{90} value from 14 days in the control to 8 days in the presence of 10 $\mu\text{g/ml}$ and to 1 day in the presence of 100 $\mu\text{g/ml}$. The addition of ammonium sulphate did not alleviate the effect of kanamycin. From this it could be concluded that ammonium sulphate enhances protein synthesis in these starved bacteria and they are subsequently killed by the addition of kanamycin. However why the other inhibitors of protein synthesis did not show a similar effect is unknown.

Figure 3.14 The effects of antibiotics on the survival of *Escherichia coli* in lake water

E. coli was grown in nutrient broth overnight at 30°C, harvested, washed and resuspended in sterile lake water. 250 ml microcosms containing 100 ml untreated lake water plus 10 µg/ml spectinomycin (Fig. 3.14a), 0.5 µg/ml streptomycin (Fig. 3.14b) or 0.5 µg/ml neomycin (Fig. 3.14c) were inoculated to give an initial viable count of approximately 10^6 cfu/ml. The flasks were incubated in the dark without shaking at 4°C, 15°C and 37°C. Viable counts were determined by surface spread plates on nutrient agar plates after incubation at 37°C for 24 h.

Fig.3.14a

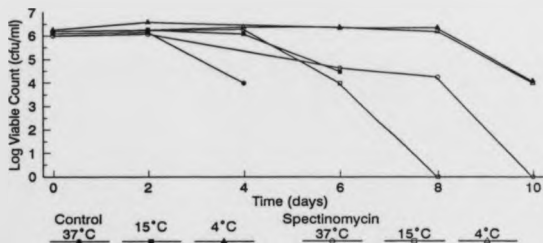


Fig.3.14b

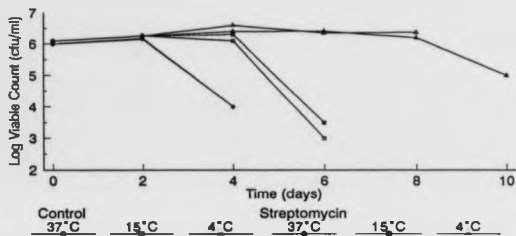


Fig.3.14c

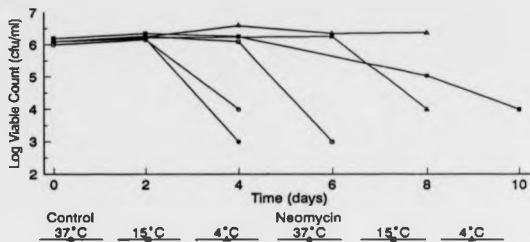


Figure 3.15 The effect ammonium sulphate plus nalidixic acid on the survival of *Escherichia coli* in lake water.

E. coli was grown in nutrient broth overnight at 30°C , harvested, washed and resuspended in sterile lake water. 250 ml microcosms containing 100 ml untreated lake water plus 100 µg/ml nalidixic acid and 100 µg/ml ammonium sulphate were inoculated to give an initial viable count of approximately 10⁶ cfu/ml. The flask were incubated in the dark without shaking at 15°C. Viable counts were determined by surface spread plates on nutrient agar plates after incubation at 37°C for 24 h.

Figure 3.16 The effect ammonium sulphate plus kanamycin on the survival of *Escherichia coli* in lake water.

E. coli was grown in nutrient broth overnight at 30°C , harvested, washed and resuspended in sterile lake water. 250 ml microcosms containing 100 ml filtered-autoclaved lake water plus 0.01 µg/ml or 0.1 µg/ml kanamycin and 100 µg/ml ammonium sulphate were inoculated to give an initial viable count of approximately 10⁶ cfu/ml. The flask were incubated in the dark without shaking at 15°C. Viable counts were determined by surface spread plates on nutrient agar plates after incubation at 37°C for 24 h.

Fig.3.15

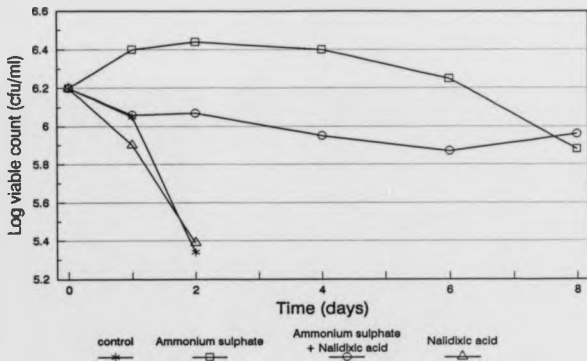


Fig.3.16

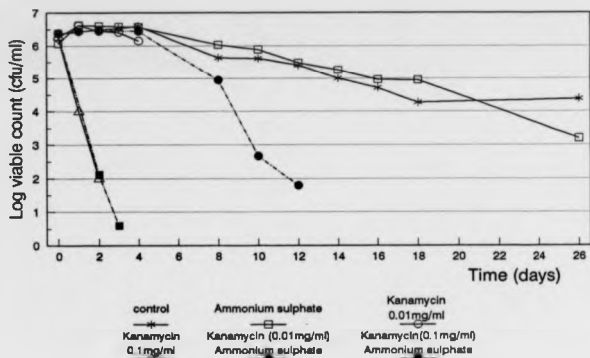


Table 3.1 The effect of antibiotics on the survival of *Escherichia coli*
in lake water (190 values, days)

Untreated lake water				
Temp.	Control	spectinomycin 10 µg/ml	streptomycin 0.5 µg/ml	neomycin 0.5 µg/ml
4°C	>10	9.0	10.0	7.0
15°C	4.8	5.3	5.0	7.6
37°C	3.0	5.2	3.0	3.0
Filtered-autoclaved lake water				
Temp.	Control	spectinomycin 10 µg/ml	streptomycin 0.5 µg/ml	neomycin 0.5 µg/ml
4°C	>10	>10	9.0	7.0
15°C	6.0	8.8	8.8	3.4
37°C	8.0	9.0	4.4	1.5

Table 3.2 The effect of spectinomycin concentrations on the survival of
Escherichia coli in lake water (190 values days)

spectinomycin (µg/ml)	Filtered-autoclaved lake water 37°C	Untreated lake water 37°C
control	12.7	5.0
10	8.5	7.3
20	9.2	5.0
30	12.0	5.0
50	4.8	0.8

Table 3.3 The effect of different concentrations of streptomycin
on the survival of Escherichia coli in lake water (190 values day)

streptomycin ($\mu\text{g/ml}$)	Filtered-autoclaved	Untreated
	lake water 37°C	lake water 37°C
control	9.0	4.0
0.5	4.6	3.0
1	3.0	2.6
1.5	5.2	2.4
2.5	0.5	5.0

Table 3.4 The effect of Ampicillin on the survival of Escherichia coli
In lake water at different temperatures (190 values days)

Ampicillin ($\mu\text{g/ml}$)		0	1	10	50	100
Filtered- autoclaved lake	15°C	28.0	28.0	28.0	28.0	3.0
	37°C	15.0	26.0	30.0	30.0	3.0
Untreated lake water	15°C	3.2	3.1	3.3	1.5	1.4
	37°C	3.0	2.3	2.2	2.2	2.2

3.3 Discussion

Several physiological and chemical factors have been considered as the main causes for the disappearance or die off faecal bacteria in aquatic bacteria. In most cases, there is no certain evidence that these factors result in actual cell death, rather the cells become non-culturable but retain their viability. When *E. coli* is released into an aquatic it has to survive under conditions where the lack of nutrients is extreme. The survival experiments reported here demonstrated that the survival of *E. coli* in lake water was inversely related to the combined influence of incubation temperature and starvation rather than to other factors in lake water.

E. coli could survive for longer at lower temperature such as 4°C rather than higher temperatures such as 37°C. Some previous work has reported that sublethal stress of *E. coli* exposed to estuarine water was more pronounced at temperatures below 10 °C (Rhodes *et al.*, 1983) and the others have shown that *E. coli* and faecal coliforms survive for shorter periods in aquatic environments at low temperatures (Mitchell, 1968; McFeters and Stuart, 1972). Nevertheless our findings support those of Flint (1987) and Lim and Flint (1990) who showed that survival of *E. coli* was a function of temperature with the longest survival times being recorded at 4°C and the shortest at 37°C in river water samples. They also reported that survival in the absence of natural microbial flora was not affected by temperatures below 25°C.

Here it was shown that 37°C was critical temperature for the survival of *E. coli*. Entry into the viable but non-culturable state was more likely to occur at this temperature than lower ones. This is consistent with the findings reported by Wolf and Oliver (1992) that entry of *Vibrio vulnificus* into the non-culturable state was highly temperature dependent. Barcina *et al.* (1986) showed that *E. coli* survived longer at 10°C than 28°C and suggested that metabolic activity of bacteria was lower at 10°C than 28°C. It is unlikely that temperature is an

important parameter alone affecting survival of *E.coli*. In considering the physiological bases for bacterial response to temperature under starvation condition, one must also take into account the relationship between temperature and the activity of other microorganisms

Some other factors, such as the influence of the composition of the growth medium as another which could influence the survival of *E.coli* in lake water. The results showed that *E.coli* grown in nitrogen-limited and phosphate-limited minimal medium could survive for extended period than cells were grown in rich medium (nutrient broth). This could be due to an early adaptation to lack of nutrient by *E.coli* grown under nitrogen and phosphate limitation. The composition of the growth medium can have other effects notably the presence of salt in the medium allows longer survival in the marine environment (Gauthier *et al.*, 1987; Munro *et al.*, 1987). Organisms grown under nutrient-limited conditions possess a markedly different physiology compared to those grown in batch cultures. The levels of catabolic enzymes increase several folds, increase intracellular cyclic AMP levels and quantitative changes in enzyme synthesis occurred. Moreover enzymes for special pathways are synthesised under starvation stress which provide the cells higher affinity for the limiting nutrients (Matin, 1979). Gottschalk (1990) reported that for faster grown culture a very rapid and dramatic response upon transfer to starvation conditions and concluded that slowly growing cells seemed much better prepared to survival periods of starvation. Phosphate starvation can cause major changes in the chemical composition the cell envelope of bacteria, thus, nutrient uptake of *E.coli* could be affected. Under condition of nitrogen limitation the synthesis of enzymes may be derepressed in order to scavenge for ammonia from the aquatic environment or produce enzymes involves in the utilisation of organic nutrient sources. They may also derepress transports systems for the uptake of various amino acids. This could be the reason why *E.coli* survive better in lake water if grown in nitrogen- and phosphate-limited media.

Cell size reduction of *E.coli* under starvation stress at different temperatures was observed in this present work by means of epifluorescence microscopy, transmission electron microscopy and Coulter counter analysis. Many researchers have shown that bacteria reduce their cell size in response to starvation stress (Baker *et al.*, 1983; Reeve *et al.*, 1984; Morita, 1985; Kjelleberg *et al.*, 1987). The experiment reported here showed that *E.coli* cells shrunk after 2 days under starvation conditions at 37°C but never became fully rounded shape. Reeve *et al.* (1984) showed that the cytoplasmic contents shrunk away from the cell envelope and some of the cells maintain their original size and shape during starvation period. This type of morphological change was also observed in the present study with some *E.coli* cells becoming folded along their edge in respond to starvation stress. After 96 days exposure to starvation, epifluorescence microscopy revealed that some *E.coli* cells did not show respiratory activity. Although the majority of cells could still respire, the plate count (viable count) was much lower than the respiring cell count. The most interesting result reported here was that alteration in cell size had very strong positive relationship with incubation temperatures. There was nearly no cell size changes at 4°C in lake water under starvation condition. However maximum cell size reduction took place when the incubation temperature was increased to 37°C. Severe loss of viability in *E.coli* was also detected at the same temperature. In conclusion, it would appear that there is a link between the viable but non-culturable and lake water (incubation) temperatures.

Becoming viable but non-culturable under the effect of starvation in aquatic environment has a potential risk to human health. This phenomenon may lead to a large underestimate of the actual number of viable organisms present if the traditional detection methods are applied. These cells still have ability to recover when suitable conditions are provided (Colwell *et al.*, 1985). *E.coli* were detected using INT reduction technique to detect respiring cells, total direct count and plate count. The results presented here imply that some *E.coli*

cells also stayed as viable but non-culturable in lake water without any nutrient supplementation for 30 days. Our experimental data also showed that there were significant differences between plate count, total direct count and respiring cell count. Although plate count started to decline at a faster rate after 18 days, respiring cell count and total direct count remained constant for up to 30 days under starvation conditions at 15°C. Quantitative comparison between respiring cell count and total direct showed that while total direct count rose at temperature 15°C and 30°C there was no increase at 4°C, the respiring cell count did not alter significantly at any incubation temperatures. This implies that cell division must take place and some cells must be losing the ability to respire. Whether this loss of ability is associated with cell division is unknown. These cells which have lost ability to respire are probably the only ones which could be considered as truly dead. Some *E.coli* do remain viable as shown by their ability to respire but are non-culturable shown by the loss in plating efficiency under these starvation conditions.

Cells subject to hypertonic solution will respond by dehydration of the protoplast. If this change is suddenly applied, damage to the cell membrane is irreversible (Sleytr and Messner, 1983). Lim (1988) has shown that in Whatman-filtered lake water the addition of 0.8 and 1 M NaCl decreased the survival of *E.coli*. However, 0.15 M and 0.5 M NaCl addition to lake water enhanced survival. Bacteria exposed to an increase in the osmolarity of their growth media responded by elevating the osmotic tension inside the cell by a build up of proline, glutamate or other organic compatible solutes (Measures, 1975; LeRudulier *et al.*, 1984b). Many authors have reported glycinebetaine and proline can play a role as osmoprotectants against the devastating effect of high osmolarity induced by high NaCl concentration (LeRudulier *et al.*, 1984b). Glycinebetaine has also been shown to be a better osmoprotectant than proline in *E.coli* at 37°C. It has been shown that the composition of outer membrane is affected by osmotic pressure and some of those effects can be reversed by the

addition of betaine or some other osmoprotectants (Barron *et al.*, 1986). Our findings have shown that severe loss of viability of *E. coli* was prevented by the addition of glycinebetaine and proline to lake water supplemented with 0.85 M. A gradual decline in numbers of *E. coli* occurred over a 10 day period then after the addition of glycinebetaine and proline. There was an increase in the colony forming units. The sudden change in the cell number probably reflects the recovery of osmotically injured cells. Similar results were obtained by Lim (1988) who showed that betaine and the proline increased survival times for *E. coli* subjected to salt concentration below 0.8 M but had no effect above this concentration. In conclusion, it is suggested that *E. coli* may remain dormant or injured due to osmotic stress and that the addition of osmoprotectants allowed the cells to repair and reverse injury, thus allowing the cells to become culturable again on agar plates.

Coliform enumeration currently the best method available for determining the safety of water, but it is generally agreed that the detection methods are influenced by environmental factors by injury due to chlorination. As a result cells lose their ability to grow on a selective media (Pelczar *et al.*, 1981; LeChevallier, 1985). It has been shown that starvation stress increases the resistance of *E. coli* to chlorine stress. The addition of 0.2 to 1.0 mg free chlorine per litre to water is usually considered to be sufficient to kill all the micro-organisms. However, here *E. coli* cells remained culturable after 6 days of incubation even in the presence of 0.29 M sodium hypochlorite in filtered-autoclaved or untreated lake water. The difference between total direct counts and plate counts may infer that some *E. coli* remained injured or stressed in the lake water. Camper and McFeters (1979) have suggested that chlorination was not effective enough for the improvement potability of water. Although, 10% *E. coli* capable of growth on selective media, 90% of injured population remained viable but non-culturable by using endogenous energy reserves until the conditions suitable for recovery occurred. In conclusion, starvation stress of

E. coli may induce resistance to stress by some chlorination cross-protection mechanism. Thus, the reliance on cell viability by plate counts for ensuring the sterility of chlorinated water supplies is questionable.

Sublethal concentrations of hydrogen peroxide, sodium dodecyl sulphate (SDS) and ethanol led to some metabolic changes in *E. coli* (Farr and Kogoma, 1991; Adamowicz *et al.*, 1991). Under hydrogen peroxide stress *E. coli* and *S. typhimurium* produce at least 30 novel proteins (Christman *et al.*, 1985) some of which confer also enhanced starvation resistance on the bacterium (Reeve *et al.*, 1984). Demple and Halbrook (1983) reported that *E. coli* cells previously exposed to hydrogen peroxide showed enhanced survival in still higher hydrogen peroxide concentrations. Although previous experiments had shown that *E. coli* survival was enhanced by pretreatment with hydrogen peroxide, our results demonstrated a loss in viability over the first few days of starvation then an increase in numbers back to the original inoculum size. This could be due to the initial stress on cells upon starvation causing loss of culturability but this injury was subsequently alleviated allowing cells to once again become culturable. This implies that hydrogen peroxide does not cause any permanent or significant damage to *E. coli*.

Adamowicz *et al.* (1991) pointed out that in SDS-grown cells, at least 4 proteins were turned on, 13 were turned off, 15 were elevated and 15 were derepressed. Kramer and Nickerson (1984) have shown that independent isolates of members of the family *Enterobacteriaceae* including *E. coli* were able to grow in SDS concentrations up to 5%. Knowing the effect of detergent on *E. coli* survival is of importance, because detergents are included in selective media. Adamowicz *et al.* (1991) have reported that *E. coli* tolerated but were unable to modify or metabolise it. Here SDS-pretreated cells lost viability to grow on agar plates when they are subsequently subjected to starvation shock. As with the cells pretreated with hydrogen peroxide they recovered the ability to grow on agar plates within a few days of starvation at 15°C. This could have been due to

the partial solubilization of the outer membrane as the cells were pretreated with SDS. This would lead to an increased starvation stress but the cells could use intracellular energy reserves or traces of nutrients in the lake water in order to repair this damage. There is no suggestion that the cells replicate in the lake water at 15°C rather that it is repair of sublethal damage.

Under appropriate conditions certain alcohols can rapidly be lethal to a range of bacteria. The mechanisms of antimicrobial activity may involve the denaturation of structural proteins or enzymes and solubilization of membrane lipids (Singleton and Sainsbury, 1987). However sublethal concentrations of ethanol resulted in induction of some proteins in *E.coli*. Here pretreatment of *E.coli* with 1% ethanol had little effect on the survival of the cells. Stressed *E.coli* once again showed a lower initial count upon transfer to the lake water microcosm but rapidly recovered the ability to grow on nutrient agar plates. Pretreatment with the three chemicals showed that their effect on *E.coli* survival was only important in the early stages of starvation as the cells rapidly recovered from the effects of pretreatment. However if the cells were starved in the continued presence of hydrogen peroxide then they were unable to recover from the stress and counts were lower than in the controls.

The presence of antibiotics may also be important factor determining the survival of *E.coli*, at least in localised microenvironments, in lake water. However to be effective the antibiotics have to be taken up by the cells and then the cells have to be metabolically active for the antibiotics to function: inhibitors of protein synthesis are unlikely to be effective as antimicrobial agents of the cells are not synthesising proteins. Antibiotics could also be helpful in increasing the survival times of *E.coli* by eliminating the natural competitors: this would be especially useful if *E.coli* was resistant to the particular antibiotics. Here the effects of the addition of ampicillin, streptomycin, spectinomycin, neomycin, nalidixic acid and kanamycin on the survival of *E.coli* in filtered-autoclaved and untreated lake water was studied.

Spectinomycin only had an effect on survival of *E.coli* at 37°C in untreated lake water where it increased the survival of *E.coli* over the controls. It had no effect in filtered-autoclaved lake water or in untreated lake water at 4°C or 15°C. Although in nutrient broth, *E.coli* is sensitive to this antibiotic it has no effect on survival on survival under these conditions. This suggests that either it is not being taken up or that it can no longer inhibit a vital function in starved cells. Only high concentration had any direct effect on *E.coli* in filtered-autoclaved lake water. Its effect at 37°C may be explained as the antibiotic having more effect on the natural microflora at this temperature, although, why it is ineffective at the more natural growth temperature is unknown. Streptomycin was also ineffective at reducing survival times in either filtered-autoclaved or untreated lake water at any temperature. Only high concentrations of 50 µg/ml (at least 50 times the MIC) were effective against *E.coli*. This implies that it is a problem with the uptake of this antibiotic which reduces its effectiveness. With neomycin the results were less easily explained. If filtered-autoclaved lake water the addition of neomycin reduced survival times but in untreated lake water it extended the survival time over that of the control. This may be explained if the neomycin is having a greater effect against the natural microflora in the untreated lake water and its effect on *E.coli* can only be observed in filtered-autoclaved lake water. The fact that it is an effective antibiotic against *E.coli* in lake water does suggest that it is able to penetrate the cell membrane even under starvation conditions. Again ampicillin had little effect on *E.coli* in either filtered-autoclaved or untreated lake water except at very high concentrations (again nearly 50 times the MIC). As there is no proof that the bacteria are undergoing cell division it is not a surprise that an inhibitor of cell wall synthesis should have little effect on the survival of *E.coli* in lake water.

Nalidixic acid is an inhibitor of DNA replication (Goss *et al.*, 1965; Dietz *et al.*, 1966) and had little or no effect on the survival of *E.coli* in lake

water suggesting that under starvation conditions little DNA replication is taking place in *E. coli*. However if ammonium sulphate, shown in Chapter 4 to increase the survival of *E. coli* in lake water was added then a difference was seen. After the addition of ammonium sulphate in the absence of nalidixic acid there was an increase in bacterial cell numbers. However, in the presence of nalidixic acid does indeed prevent DNA synthesis under starvation conditions. Again the only effective concentration of nalidixic acid was over 10 times the MIC in nutrient broth. Similarly kanamycin used at 50 times the MIC was effective in decreasing the μ_{90} value in filtered-autoclaved lake water. As this is an inhibitor of protein (Spirin, 1973) synthesis as is streptomycin, neomycin and spectinomycin which had little effect then it is probable that this antibiotic was taken up more efficiently than the others.

Although the results with the antibiotic additions are far from clear they suggest that even under starvation conditions the cells still possess the ability to synthesise DNA and protein. However the startling result is the increased resistance of the cells to the antibiotics. All were effective only at many times the concentration usually considered to be MIC (ascertained in nutrient broth). This suggests that either the cells are not taking up the antibiotic efficiently and the increased dose is needed because penetration to the interior of the cell is simply by diffusion or the possibility that starvation renders bacteria more resistant to the effects of antibiotics in the same way that there is cross-protection afforded between heat and oxidative shock.

**Chapter IV. Factors Affecting the Survival of *Escherichia coli* in
Lake Water**

4.1. Introduction

4.1.1 The effect of nutrients on survival

Many researchers have suggested that the reasons for the reduction in the population size of bacterial species released into fresh waters were temperature (Flint, 1987; Rhodes and Kator, 1988), pH (Zaidi *et al.*, 1988), solar radiation (Barcina *et al.*, 1986), the presence of toxins (Bloem and Bar-Gilissen, 1989), protozoan predation (Habte and Alexander, 1978; Alexander, 1981; McCambridge and McMeekin, 1981), bacteriophages (Bergh *et al.*, 1989; Proctor and Fuhrman, 1990) and starvation stress (Morita, 1982; Flint, 1987). Bacterial survival also depends on cells deriving essential nutrients from the environment. These nutrients enable the cells to build up necessary material for cell biomass and for the production of biologically utilizable energy for such functions as cellular motility, the secretion of digestive extra cellular enzymes and chemotaxis (Gottschalk, 1979a). In aquatic systems, the indigenous microflora takes up nutrient and energy sources by very efficient transport mechanisms (Button, 1985). In addition, in the natural environment, bacteria reproduce and grow in mixed culture on miscellaneous sources of carbon, nitrogen, phosphorus, and sulphur. For aquatic micro-organisms, survival is dependent upon the rapid utilisation of substrates as they became available. In the context of survival, it is the total range of mechanisms by which microbial cells can select, take up and utilise carbon sources that is significant (Paigen *et al.*; 1970). It has been shown that the addition of organic and inorganic nutrient supplements to water can alter the survival times of coliforms in the marine and fresh water environment (Carlucci and Pramer, 1960; Lim and Flint, 1989). This can have serious implications for the use of coliforms as indicator organisms.

4.1.1.1 Carbohydrates

Carbohydrates enter bacterial cells by a variety of different mechanisms. Glycerol, for example, is believed to cross the cytoplasmic membrane by facilitated diffusion, an energy-independent process (Hayashi and Lin, 1964; Lin, 1970). On the other hand, disaccharides, such as maltose, lactose and melibiose, pass through the Gram-negative bacterial cell membrane by several energy-dependent active transport processes. Maltose is transported by a periplasmic binding protein system which appears to be dependent on a chemical form of energy, such as ATP (Berger, 1973). Lactose and melibiose, on the other hand, are transported by two distinct permease systems, neither of which requires chemical energy nor a periplasmic binding protein for activity. Instead an electropotential gradient across the membrane is used (West and Mitchell, 1972). In brief, it has been shown that some carbohydrates may be transported into cells by an energy-independent process or by a energy-dependent concentration mechanism. The carbohydrates remain unchanged or may be phosphorylated during the uptake process into the cytoplasmic compartment of the cell (Dilla *et al.*, 1980). The sugars and sugar derivatives which *E. coli* can utilise have been examined, and the permeases and other mechanisms by which these sugars are taken up have been investigated (Gottschalk, 1976b). However, it is unlikely that *E. coli* can successfully compete for carbohydrates in natural environments as the natural microflora should be able to take up the nutrients faster at the ambient temperatures.

4.1.1.2 Carboxylic acids

In aquatic environments transport systems for carboxylic acids, which are also carbon sources, have received relatively little attention. Many organic acids are not only a source of carbon and energy, but also vital intermediates in gluconeogenesis and anaplerosis (Kornberg, 1969; Sanwal, 1970). A variety of

organic acids can be utilised by microorganisms as electron donors and carbon sources. Some microorganisms cannot take up organic acids directly from their environment. Therefore organic acids are converted to other forms by specific enzymes prior to uptake. DL-lactate has been shown to be actively transported into a L-lactate oxidase mutant of *E. coli* but an unusual method of accumulation was observed (Brown, 1972). Pyruvate has been shown to be actively transported into membrane vesicles of *E. coli* energised with reduced phenazine methosulphate but not into vesicles of *Bacillus subtilis* or of a *Pseudomonas* species (Lo *et al.*, 1974). In *E. coli* K12, acetate and propionate appear to share a common uptake system. The uptake of C₄-dicarboxylates (succinate, fumarate, L-malate and L-aspartate) by *E. coli* has been shown (Kay and Kornberg, 1969).

4.1.1.3 Nitrogen sources

Nitrogen is another important nutrient required for bacterial survival. It has been shown that nitrogen sources can affect *Escherichia coli* survival in natural aquatic environments (Lim and Flint, 1989). It is known that nitrogen occurs naturally in many different forms e.g. ammonia nitrate, nitrite, dinitrogen and nitrogen-containing organic compounds. Ammonia can be taken up by practically all microorganisms and nitrate is used by a significant number of microorganisms. However, nitrate has to be reduced to ammonia before it is combined into organic compounds (Gottschalk, 1986). In many natural environments, the concentration of usable nitrogen sources is insufficient to support optimum growth of organisms. Nitrogen concentration is usually very low in fresh and marine environments rarely exceeding a few parts per million except in polluted areas. In aquatic sediments the concentration of ammonium ions can be higher (Cole and Brown, 1980). Although there is a low concentration of ammonium ions in aquatic environments, some bacteria can store ammonium inside the cell for further utilisation (Youth and Pengra, 1966).

In connection with this, three different *E. coli* strains were studied by Stevenson and Silver (1977) who showed that *E. coli* strains concentrated methylammonium intracellularly up to 100 fold, while *B. subtilis* did not take up this compound. The ability to store a nitrogen reserve might give bacteria an advantage when the natural microbial flora is present and nitrogen is limiting. Amino acids are also used as nitrogen sources by bacteria in many environments. Many bacteria can synthesise the twenty amino acids necessary for protein biosynthesis, utilising inorganic ammonium salts as a nitrogen source. In general biosynthesis of amino acids is strictly controlled by mechanisms such as end product regulation (Umberger, 1969) and most microorganisms do not overproduce them. These facts imply that the control mechanisms are physiologically effective and also that the bacterial envelope is a highly impermeable barrier for the amino acids which are synthesised endogenously. On the other hand, bacteria can also utilise amino acids from the medium by actively taking them up (Kepes and Cohen, 1962). Many bacteria maintain an internal amino acid pool that is involved in the biosynthesis of proteins and various nitrogen containing, organic metabolites (Britten and McClure, 1962ab; Holden, 1962). The amount and composition of the pool of amino acids are important factors in the regulation of microbial growth and also survival of bacteria in the environment (Tempest *et al.*, 1970). It has been shown that a lack of amino acids causes cessation of growth and the initiation of intracellular turnover of proteins (Tempest *et al.*, 1970). Under starvation conditions, some amino acids could also become limiting necessitating the turnover of proteins seen in the initial stage of starvation (Reeve *et al.*, 1984).

4.1.1.4 Sewage effluent

Lakes, rivers, streams and the other surface waters can be contaminated with sewage effluent. It is known that this is the main source of coliform bacteria in estuaries and rivers (White and Godfree, 1985). *E. coli* is one of the important

indicator organisms for faecal pollution. Flint (1987) suggested that sewage effluent is not only the source of bacteria but also a source of nutrients which are optimally utilised by *E. coli* when the temperature are suitable. Flint (1987) showed that *E. coli* survival was slightly enhanced in samples taken from a river subjected to sewage effluent than samples taken above the point of effluent input. Furthermore, it was shown that sewage effluent was successfully utilised by *E. coli* cells and led to increased number of cells at temperatures greater than 4°C. This could be due to very low cell metabolic activity at that temperature. Lim (1989) showed that thermally damaged *E. coli* cells recovered more rapidly in sewage polluted lake water than in unsupplemented lake water. He also showed that *E. coli* survival was increased by the addition of synthetic sewage to the lake water.

4.1.2 The effects of predation on the survival of *E. coli*

Protozoan predation is known to be an important factor in the declining number of bacteria native to fresh waters (Barcina *et al.*, 1986; Rhodes and Kator, 1988). Gurijala and Alexander (1990) suggested that protozoa, predominantly microflagellates were responsible for the rapid decline in cell number at 30°C. The longer survival at lower temperatures could be due to slower protozoan replication at these temperatures. Similar results were obtained by Flint (1987) who suggested that *E. coli* survived longer at temperature 4°C than other temperatures in river water but suggested that predation was not an important factor. In addition, Andersen and Fenchel (1985) observed that the disappearance of *E. coli* was affected by the seasonal variation; again implying that water temperature was important. A common method of studying the effects of protozoa on bacteria is to employ eukaryotic inhibitors such as cycloheximide and nystatin to inhibit protozoan activity. Although some researchers have used these inhibitors to study ecological role of protozoa, others have recommended being cautious when using specific inhibitors. Sanders and Porter (1986) showed

that several common freshwater protozoans continued to swim and ingest bacterium-size latex beads in the presence of cycloheximide and amphotericin B and also showed that the ingestion rates of several ciliates were unaffected by the presence of eukaryotic inhibitors. Contrary to the above suggestions, *E. coli* survived for a different length of time in different lakes when protozoa were suppressed by the addition of 250 μg cycloheximide and 30 μg of nystatin per ml (Barcina *et al.*, 1986; McCambridge and McMeekin, 1979; Gurijala and Alexander, 1990). As a result of this treatment, protozoa were not detected after day one. Gonzalez *et al.* (1992) suggested that in natural aquatic systems, predation would be caused by protozoa or by other agents such as predatory bacteria (e.g., *Bdellovibrio* s.) and bacteriophages. The size fractionation of microbial species has also been used to estimate the effects of microbial grazing on survival in water samples. Filtration through nucleopore filters with a pore size of 1 μm will remove most protozoa although some microflagellates can pass through and bloom (Cynar *et al.*, 1985). Sanders and Porter (1986) showed that filtration through a 63 μm mesh had no effect on changing bacterial density relative to untreated control sample, indicating that microzooplanktons were responsible for most of the bacteriivory. Mallory *et al.* (1983) found that neither filtration nor the inhibitors alone removed all protozoa from the water body.

Inoculum size of bacterial population was believed to be another parameter affecting bacterial survival. Protozoa which can grow by feeding on various bacterial species are not able to reduce the density of a bacterial prey below a certain population size (Sinclair and Alexander, 1989; Habte and Alexander, 1978). It has been proposed that the predator will eliminate a prey species from a natural environment when an alternative prey is present at concentrations above the threshold number for active feeding by the predator

and when the rate of growth of the prey is less than the rate of predation (Mallory *et al.*, 1983).

4.2. Results

4.2.1. The effects of nutrient sources on the survival of *E. coli*

4.2.1.1 The effect of carbohydrates on the survival of *E. coli*

In these experiments monosaccharides and disaccharides were used as carbon sources to amend untreated and filtered-autoclaved lake water samples. Representative graphs show the survival of *E. coli* at 15°C (Fig 4.1) and 30°C (Fig 4.2) in untreated lake water amended with glucose, lactose and sucrose. In some cases the *E. coli* was able to increase in numbers prior to the decline more commonly seen. This suggests that *E. coli* would be able to grow in the lake water after the addition of suitable nutrient sources. Fig 4.3 shows the effects of amendment with maltose on the survival of *E. coli* in untreated water at both temperatures. There was little increase in the t_{90} values for survival with any amendment (Table 4.1). In untreated lake water at 30°C fructose gave the largest increase in t_{90} value but the other carbon sources had little effect. In untreated lake water at 15°C maltose, lactose and glucose increased the t_{90} values but the other carbohydrates had little effect. Any changes in survival ability could be due to effects directly on *E. coli*, i.e. the ability of the cells on metabolise the substrate, or indirectly through effects on the other components of the microflora with which *E. coli* is competing for essential nutrients. In filtered-autoclaved lake water only lactose increased the t_{90} value at 30°C although *E. coli* could grow on lactose, fructose, mannose, glucose and galactose under these conditions. At 15°C the t_{90} values were reduced compared to the control by all the carbon sources except lactose. This may be an example of substrate-accelerated death reported for some other starvation situations (Postgate, 1961). This could be due to the accumulation of the carbohydrate

source by the bacterial cells without metabolising it because of the absence of other essential nutrients needed for the generation of new biomass.

4.2.1.2 The effects of carboxylic acids on the survival of *E. coli*

Carboxylic acids were used as carbon sources to amend untreated and filtered-autoclaved lake water. The inoculated flasks were incubated at 15°C and 30°C. The results are shown in representative graphs in Figures 4.4, 4.5, 4.6. Table 4.2 shows the results as t_{90} values for the complete range of carboxylic acids which were used. In untreated lake water at 30°C amendment with carboxylic acids had little effect on the survival of *E. coli*. Only fumarate and citrate had any real effect extending the t_{90} value from 2.2 days to 3.2 days. All the other carboxylic acids only increased the t_{90} values slightly. At 15°C amendment of untreated lake water with carboxylic acids had a more apparent effect, with all the amendments except succinate and formate extending the t_{90} values from 4.5 days to at least 7 days and up to 10 days in case of acetate. In these microcosms there was also an increase in the numbers of *E. coli* over the first 2 days of incubation with lactate and acetate. This suggests that *E. coli* can utilise these carboxylic acids as carbon sources under these conditions and in the presence of the microbial flora of the lake water. In filtered-autoclaved lake water at 30°C survival was enhanced by all the carboxylic acids used as amendments with the t_{90} value being increased from 39 days in the controls to more than 60 days in the amended samples. At 15 °C there was no change in the t_{90} value which was in excess of 60 days in the control and the test microcosms. Under these conditions *E. coli* was able to utilise a number of the carboxylic acids most notably succinate, pyruvate, lactate, acetate, leading to an increase in the size of population at both 15°C and 30°C.

Figure 4.1 The effect of carbohydrates on the survival of *Escherichia coli* in untreated lake water at 15°C.

Figure 4.2 The effect of carbohydrates on the survival of *Escherichia coli* in untreated lake water at 30°C.

E.coli was grown in nutrient broth overnight, harvested, washed and resuspended in lake water. 100 ml microcosms were inoculated to give an initial viable count of approximately 10^6 cfu/ml. The flasks were incubated at 15°C (Fig 4.1) and or 30°C (Fig 4.2) in the dark without shaking. The lake water was amended with D-glucose, sucrose or lactose at 2 mg C/l final concentration. Viable count were determined using surface spread plates incubated at 37°C overnight.

Fig.4.1

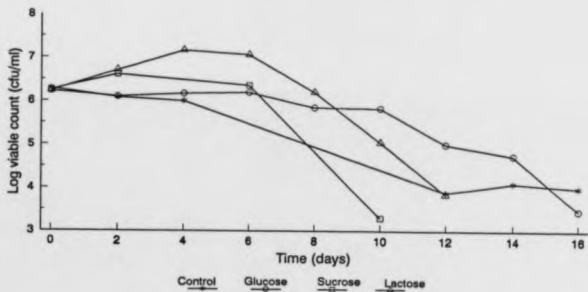


Fig. 4.2

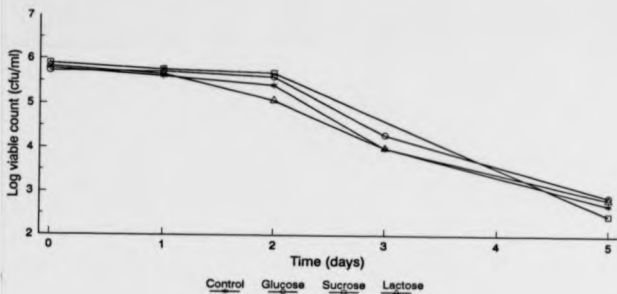


Figure 4.3 The effect of maltose on the survival of *Escherichia coli* in untreated lake water at 15°C and 30°C.

E.coli were grown in nutrient broth overnight, harvested, washed and resuspended in sterile lake water. 100 ml lake water microcosms were inoculated to give an initial viable count of approximately 10^6 cfu/ml. Untreated lake water was amended with maltose at a final concentration of 2 mg C/l.

Fig. 4.3

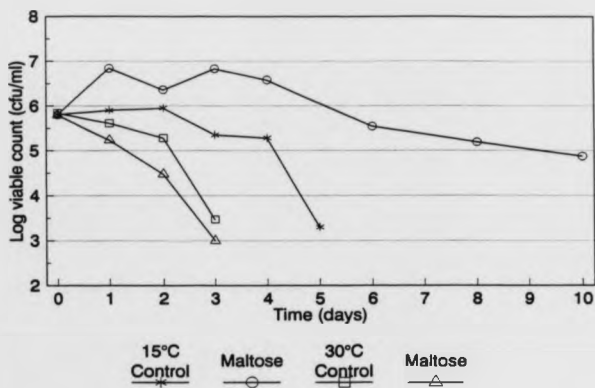


TABLE 4.1 Survival of *Escherichia coli* in carbohydrate-amended lake water

Carbohydrates	Untreated Lake Water				Filtered-autoclaved Lake Water			
	control	15°C	control	30°C	Control	30°C	control	15°C
Mannose	5.0	5.5	2.2	3.0	25	27	>80	50
D-Fructose	5.5	5.7	2.2	5.7	25	25	>80	70
Galactose	5.0	5.5	2.2	3.8	39	25	>80	60
Maltose	5.0	10.0	2.2	1.5	25	23	>80	60
Sucrose	5.0	5.8	2.2	2.8	25	25	>80	50
Lactose	5.5	8.2	2.2	1.5	25	44	>80	80
D-Glucose	5.5	10.0	2.2	2.2	25	25	>80	69

Values given are t90 values in days. This is the time taken for a one-log drop in viable count from the original inoculum size.

Control values for unamended lake water;

Untreated 30°C 2.2 d 15°C 5.5 d

Filtered-autoclaved 30°C 25 d 15°C >80 d

The experiments were terminated after 80 d incubation.

Figure 4.4 The effect of carboxylic acids on the survival of *Escherichia coli* in untreated lake water at 15°C.

Figure 4.5 The effect of carboxylic acids on the survival of *Escherichia coli* in untreated lake water at 30°C.

Figure 4.6 The effect of carboxylic acids on the survival of *Escherichia coli* in filtered-autoclaved lake water at 15°C.

E.coli was grown in nutrient broth overnight, harvested, washed and resuspended in lake water. 100 ml microcosms were inoculated to give an initial viable count of approximately 10^6 cfu/ml. The flasks were amended with the following carboxylic acids at the final concentration given; fumarate, succinate, formate and the pyruvate at 1 mg-C/l, citrate, lactate and acetate at 1.5 mg-C/l and propionate at 2 mg C/l. Viable counts were determinate using surface spread plate, incubated at 37°C overnight. The flasks were incubated in the dark without shaking at 15°C and 30°C

Fig.4.4

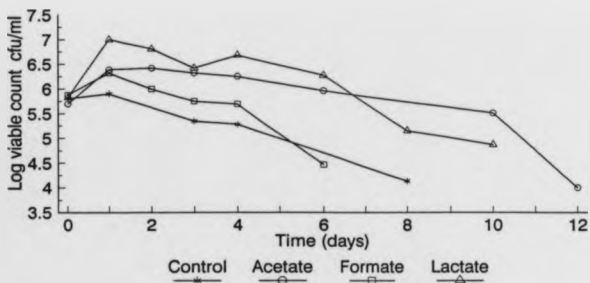


Fig.4.5

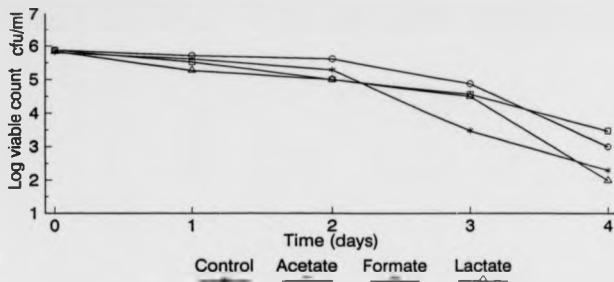


Fig.4.6

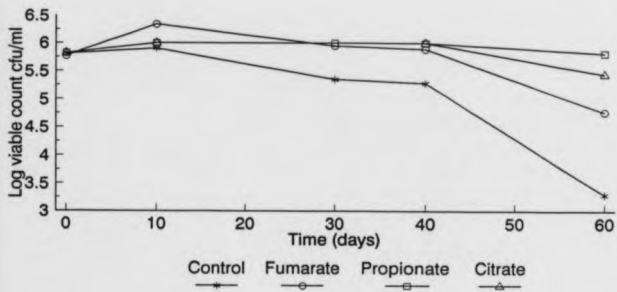


TABLE 4.2 Survival of *Escherichia coli* in lake water amended with carboxylic acids

Carboxylic Acids	Untreated lake water				Filtered-Autoclaved Lake water	
	Control	15°C	Control	30°C	Control	30°C
L-Lactate	4.5	10.0	2.2	2.4	39	>60
Fumarate	4.5	7.5	2.2	3.2	39	55
Acetate	4.5	10.0	2.2	3.1	39	>60
Propionate	4.5	8.0	2.2	2.4	39	>60
Citrate	4.5	7.0	2.2	3.2	39	>60
Succinate	4.5	4.0	2.2	3.0	39	>60
Formate	4.5	5.5	2.2	2.4	39	>60
Pyruvate	4.5	9.0	2.2	2.4	39	>60

Values given are t90 values in days. This is the time taken for a one-log drop in viable count from the original inoculum size

Control values for unamended lake water;

Untreated 30°C 2.4 to 3.1 d 15°C 4.5 d

Filtered-autoclaved 30°C 39 d 15°C 60 d

The experiments were terminated after 60 d incubation.

4.2.1.3 The effect of amino acids on the survival of *E.coli*

Amino acids are one of the important growth factors for living organisms, hence they could play a critical role in the survival of bacteria in any environment. *E.coli* survival was examined in untreated and filtered-autoclaved lake water to which amino acids were added individually. According to the results presented here *E.coli* survival was enhanced by the addition of L-glutamine, glycine, L-proline and L-alanine to the filtered-autoclaved lake water at 15°C and 30°C. At the same time L-glutamine, L-alanine and proline enabled *E.coli* to grow efficiently and the population size increased almost 1 log in an average of 4 days incubation time (Fig 4.7a). T₉₀ values were also increased to greater than 30 d by the addition of L-glutamine and glycine, 24 d with L-alanine and 18 d by proline addition to the filtered-autoclaved lake water at 30°C. Survival was increased profoundly by the addition of the above mentioned amino acids to the filtered-autoclaved lake water at 15°C (Fig 4.7b). After 34 d there was little decline in number of bacteria in all samples at 15°C and the cell number remained constant. In untreated lake water at 15°C, survival was increased by the addition of L-alanine, glycine and L-proline but faster loss of viability was observed in case of L-serine and glutamine addition over the control (Fig 4.8a). In general the specific rate of decay was also higher when L-serine, glutamine or glycine had been added to the lake water at 15°C. However L-alanine and L-proline extended survival over 5 days in the control. This could be due to a direct effect on *E.coli* or the promotion of growth in the competing microflora. In untreated lake t₉₀ values did not show any important changes at 30°C by the addition of any of the above mentioned amino acids (Fig 4.8b). The data for amino acids are presented in Table 4.3. These did not alter *E.coli* survival in untreated lake water by significant amounts. However some of them showed small increases in the t₉₀ value of between 0.5 to 2 d at 15°C. On the other hand at 30°C there was almost no alteration in terms of survival.

Figure 4.7a The effect of amino acids on the survival of *Escherichia coli* in filtered-autoclaved lake water at 30°C.

Figure 4.7b The effect of amino acids on the survival of *Escherichia coli* in filtered-autoclaved lake water at 15°C.

E. coli was grown in nutrient broth overnight, harvested, washed and resuspended in sterile lake water. 100 ml microcosms were inoculated to give initial population size of approximately 10^6 cfu/ml. The lake water was amended with amino acids at final concentrations of 2 mg/l. The flasks were incubated in the dark at 15°C or 30°C without shaking. Viable counts were determined by surface spread plates incubated at 37°C overnight.

Fig 4.7a

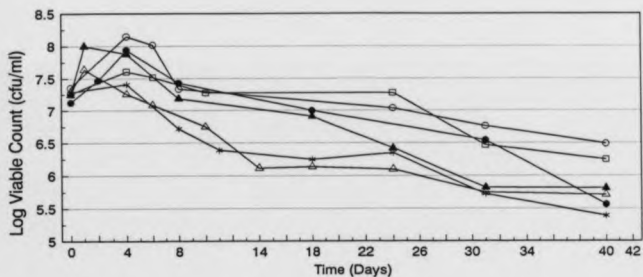


Fig. 4.7b

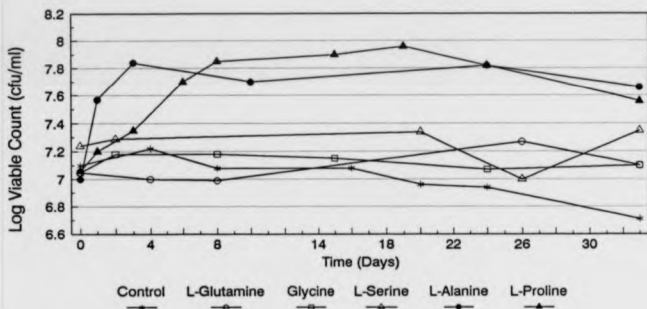


Figure 4.8a The effect of amino acids on the survival of *Escherichia coli* in untreated lake water at 15°C.

Figure 4.8b The effect of amino acids on the survival of *Escherichia coli* in untreated lake water at 30°C.

E. coli was grown in nutrient broth overnight, harvested, washed and resuspended in sterile lake water. 100 ml microcosms were inoculated to give 10^6 or 10^7 cfu/ml. The lake water was amended with amino acids at final concentrations of 2 mg/l. The flasks were incubated in the dark at 15°C or 30°C without shaking. Viable counts were determined by the surface spread plates incubated at 37°C overnight.

Fig.4.8a

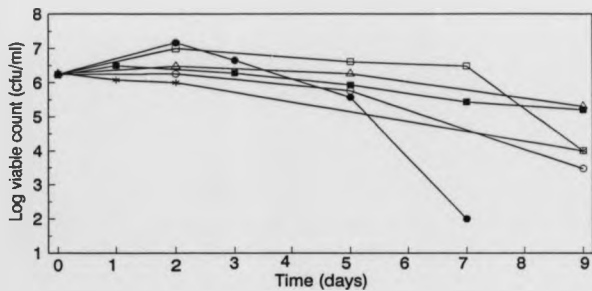


Fig.4.8b

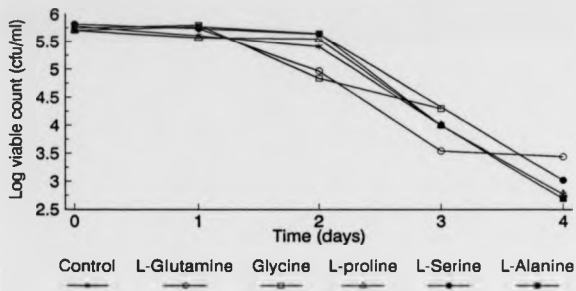


TABLE 4.3 Survival of *Escherichia coli* in untreated lake water amended with amino acids

Amino acids	Untreated lake water			
	Control	15°C	Control	30°C
L-Lysine	4.0	5.7	3.0	3.0
L-Methionine	4.0	6.0	3.0	2.8
L-Glutamate	4.0	6.0	3.0	3.0
L-Tyrosine	4.0	4.5	3.0	2.4
DL-Phenylalanine	4.0	6.0	3.0	2.6
L-Arginine	4.0	5.0	3.0	2.3
L-Asparagine	4.0	5.0	3.0	3.5
L-Cysteine	4.0	4.7	3.0	3.0
L-Histidine	4.0	6.0	3.0	3.0
DL-Tryptophane	4.0	4.5	3.0	2.2

Values given are t90 values in days. This is the time taken for a one-log drop in viable count from the original inoculum size.

Control values for unamended lake water;

Untreated 30°C 3.0 d 15°C 4.0 d

The experiments were terminated after 10 d incubation

4.2.1.4 The effect of nitrogen sources on the survival of *E.coli*

Ammonium sulphate, ammonium nitrate, potassium nitrate, sodium nitrate, and casein were used as nitrogen amendments in microcosms at concentrations from 50 to 500 mg-N/l in filtered-autoclaved and untreated lake water. The results are represented graphically in Figs 4.9 and 4.10. Most nitrogen sources have a substantial effect on the survival of *E.coli* in untreated lake water at 15°C. Ammonium sulphate and ammonium nitrate increased t_{90} values from approximately 3 days to more than 25 days in untreated lake water. Potassium and sodium nitrate had no comparable effect suggesting that it was the ammonium ion component which was the active factor. At 30°C there was only a slight increase in the t_{90} value with the same concentrations of ammonium sulphate and nitrate.

Casein also increased the t_{90} values by more than 20 days in untreated lake water at 15°C but again only slightly increased the t_{90} value at 30°C. *E.coli* could utilise casein as a growth substrate at both 30°C and 15°C leading to an increase in viable count of almost one log. Fig 4.10 clearly shows that at 15°C *E.coli* becomes virtually undetectable in untreated lake water within 10 days but after the addition of casein as a nitrogen source bacterial counts persist for more than 25 days and can increase even in the presence of natural microbial flora.

4.2.2 The effects of synthetic sewage addition on the survival of *Escherichia coli*

The survival of *E.coli* in sewage-amended lake water was found to be temperature dependent. The addition of 5 to 25 % (v/v) synthetic sewage resulted in *E.coli* cell numbers increasing in filtered-autoclaved lake water and also prolonged the survival of *E.coli* in untreated lake water at 15°C. The

results are expressed graphically in Fig 4.11 and 4.12 for 15°C; the results for the other temperatures were essentially the same and are not shown graphically. There was no growth of *E. coli* in sewage-amended in lake water at 4°C and the t₉₀ value remained unchanged. This suggests that *E. coli* are metabolically inactive at 4°C and cannot utilise the nutrients supplied at this low temperature. This finding was supported by Flint (1987) who showed that in river water subjected to sewage pollution there was no growth of *E. coli* at 4°C.

In the absence of amendments with synthetic sewage *E. coli* was not able to remain viable for more than 6 days in the presence of the natural microbial flora of the lake. In the sewage-amended samples viability was maintained for a longer period of time (Fig 4.12). At 15°C it is possible that the addition of sewage to the lake water could be advantageous to *E. coli* allowing the bacteria to grow even in the presence of natural microbial flora. The bacteria were able to increase in numbers at 15°C in filtered-autoclaved lake water again showing that it is possible for *E. coli* to grow at 15°C under these conditions. The growth was proportional to the concentration of synthetic sewage added (Fig 4.11). The increase in numbers in untreated lake water was not as great as that seen in the filtered-autoclaved lake water but the numbers of bacteria did not decline as seen in the control. This pattern was also seen at 25°C and 30°C.

It is not surprising that *E. coli* grew in sewage-amended lake water. The longer survival in the presence of the natural microbial flora in sewage supplemented lake water indicated that the main cause for the decline in *E. coli* numbers in lake water is competition for nutrients. When sewage was added to the lake water *E. coli* was probably able to compete successfully with the natural microflora possible eliminating some competing bacterial species and growing

Figure 4.9 The effect of casein on the survival of *Escherichia coli* in untreated lake water at 15°C.

Figure 4.10 The effect of ammonium sulphate and ammonium nitrate on the survival of *Escherichia coli* in untreated lake water at 15°C.

E.coli was grown in nutrient broth overnight, harvested, washed and resuspended in sterile lake water. 100 ml microcosms were inoculated to give an initial viable count of approximately 10^6 cfu/ml. The lake water was amended with casein at concentrations of 50, 100 and 150 mg/l, with ammonium sulphate at 50 and 500 mg-N/l or with ammonium nitrate at 100 and 300 mg-N/l. The flasks were incubated at 15°C in the dark without shaking. Viable counts were determined using a surface spread plates, incubated at 37°C overnight

Fig.4.9

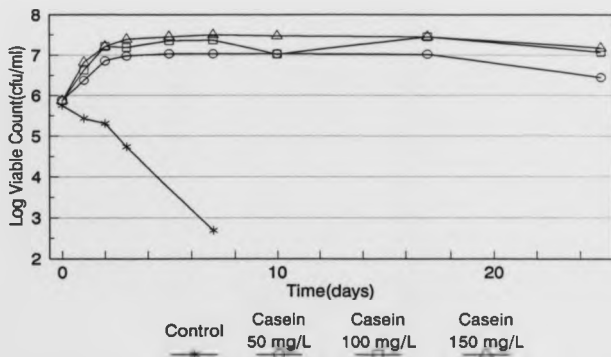


Fig.4.10

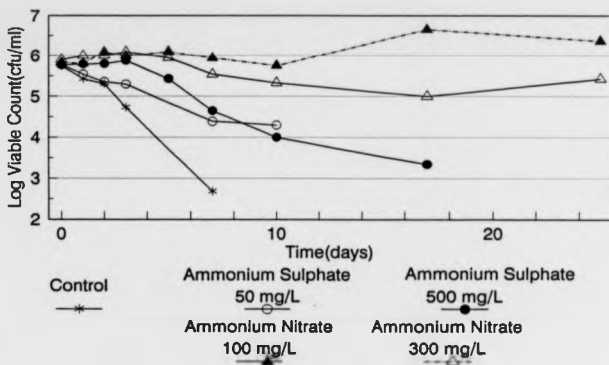


Figure 4.11 The effect of synthetic sewage on the survival of *Escherichia coli* in filtered-autoclaved lake water at 15°C.

Figure 4.12 The effect of synthetic sewage on the survival of *E.coli* in untreated lake water at 15°C.

E.coli was grown in nutrient broth overnight, harvested, washed and resuspended in sterile lake water. 100 ml microcosms amended with synthetic sewage at final concentrations ranging from 5 to 25 % (v/v) were inoculated with approximately 10^6 cfu/ml. The flasks were incubated at 15°C in the dark without shaking. Viable counts were determined by surface spread plates, incubated overnight at 37°C.

Fig. 4.11

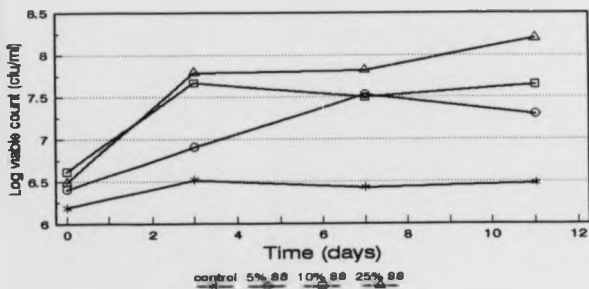
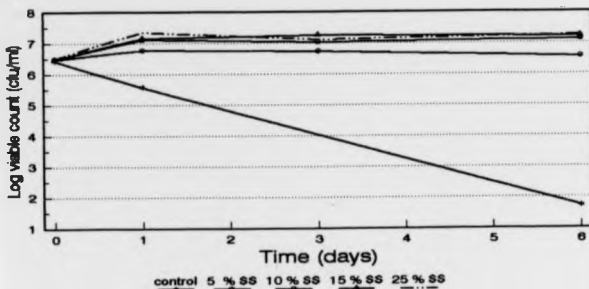


Fig. 4.12



faster than the rate at which bacteria were preyed by protozoa as suggested by Gurijala and Alexander (1990).

4.2.3 The Effect of Natural Competitors and Protozoan Grazing on the Survival of *Escherichia coli*

4.2.3.1 The effects of predation on the survival of *Escherichia coli*

As has been shown above loss of viability in *E.coli* occurred when the cells were inoculated in to lake water in the presence of the natural microbial flora, i.e. in untreated water. Repeated results suggested that *E.coli* could not survive for long periods of time in untreated lake water at temperatures where the cells would be metabolically active. Generally survival was for less than 10 days in untreated lake water at 15°C and even less at higher temperatures. The effects of temperature on survival have also been noted previously by Flint (1987) and Lim and Flint (1989). Fig 4.12 shows that *E.coli* survives for longer at low temperatures in filtered- autoclaved and untreated lake water probably due to the low metabolic activity of the cells including the competitor organisms of the natural microbial flora. *E.coli* could survive for up to 260 days in sterile river water (Flint, 1987) and for more than 200 days in the experiments with sterile lake water reported here (Fig 4.14). However survival at comparable temperatures nearly exceeded 10 days in the presence of the indigenous microflora. In untreated lake water the t₉₀ values for survival were 2.4 d at 37°C, 4.9 d at 15°C and 8.2 d at 4°C. In filtered-autoclaved lake water these were extended to 5.1 d at 37°C and to more than 200 d at all other temperatures. These results imply that temperature was one of the main factors affecting survival of *E.coli* in untreated lake water but the survival of *E.coli* in untreated lake water either through predator or competition for nutrients.

In order to investigate the importance of predation to the rapid decline in the numbers of *E.coli* in untreated lake water, a series of experiments were conducted using different method of filtration, inhibitors of eukaryotic activity and the addition of bacteriophage to compare the effects of the protozoan and the viral predators and the bacterial competitors on the survival of *E.coli* compared to untreated controls. Metabolic inhibitors such as cycloheximide and nystatin which have been reported to selectively inhibit grazing protozoa and have previously been used to measure the rates of bacterial growth and protozoan grazing activity in lake water microcosms. This method assumes that the net change in bacterial abundance when one of the factors is removed is a direct measurement of the remaining process. Here the addition of cycloheximide at 300 $\mu\text{g/ml}$ had little effect on the survival of *E.coli* in untreated lake water at 15°C and 30°C (Fig 4.15). The addition of nystatin at 250 $\mu\text{g/ml}$ did increase survival at 15°C but not significantly at 30°C. Nystatin increased the tg value from 3.1 d to 5.7 d at 15°C but only from 5.6 to 6.4 at 30°C.

These results suggest that predation plays a minor role in the disappearance of *E.coli* in these samples. If protozoa predation had been a major factor then *E.coli* would have been expected to survive for longer periods of time in the presence of eukaryotic inhibitors which would interfere with the functioning of protozoa. This was clearly not the case. It is possible that the concentration of inhibitor used was not high enough but previous reports had suggested grazing activity would be inhibited through interference with protein synthesis (Sanders and Porter, 1986). Protozoa would be expected to be affected by the temperature as much as the bacterial cells. They more metabolically active at temperatures around 15°C to 25°C, the natural temperatures which they encounter. Some of the small differences seen in survival at different temperatures could be a reflection of the changing rate of predation with

temperature. However in our system we believe that predation by protozoa is of minor significance in the disappearance of *E. coli* from lake water.

The small effect of protozoa on the survival of *E. coli* was also supported in experiments conducted in water samples subjected to different filtration treatments. Water was filtered through Whatman filter paper to remove protozoa and particulate material, and through Millipore filters with pore size of 0.45 μm and 0.2 μm to remove all bacterial and protozoan cells. Untreated water was used as a nonsterile control and the filtered-autoclaved lake water as a sterile control. The results are expressed graphically in Fig 4.16 for survival at 30°C and Figure 4.17 at 15°C. Filtration through Whatman filter paper had little effect on the survival of *E. coli* compared to the untreated control at either temperature. Filtration through a Millipore filter with either pore size, however, increased the survival time at 15°C (t_{90} values increased from 3.3 d in the nonsterile control to >5 d in the sample filtered through the 0.45 μm filter and > 20 d in the sample filtered through the 0.2 μm filter). At 30°C although the t_{90} values were little different between the untreated control and the Millipore-filtered samples the residual population after 5 d of incubation was higher in the 0.45 μm -filtered sample and higher still in the 0.2 μm -filtered sample. At both temperatures complete sterilisation of the sample by autoclaving filtered water led to the longest survival times. In both experiments the t_{90} value was in excess of 20 d, the length of the experiment. These results were similar to those shown previously by Enzinger and Cooper (1976) and McCambridge and McMeekin (1981) where filtration through 0.45 μm and 0.8 μm filters led to increased survival of *E. coli* in lake and river water.

The decline in the numbers of *E. coli* in the samples which had been subjected to Millipore filtration could have been due to the effects of lytic bacteriophage. The major difference between filtered-autoclaved sterile water and Millipore-filtered water was considered to be the presence of bacteriophage

in the latter samples. Consequently the survival of *E.coli* was assessed in microcosms to which bacteriophage were added at the same time as the *E.coli* was inoculated. The results are shown in Fig 4.18. The largest decline in bacterial numbers was seen when the filtered-autoclaved lake water was amended with 10^6 bacteriophage particle per ml. The reduction in numbers of *E.coli* in the other samples was proportional to the initial titre of the bacteriophages added. There was little or no reduction in the viable count in samples which had less than 10^4 phage particles per ml added. High numbers of bacteriophages are not expected in lake water and as a consequence bacteriophages may have little effect on the decline of *E.coli* in natural lake waters. Bergh *et al.* (1989) and Proctor and Furhman (1990) have also observed the minor role which bacteriophage play in the disappearance of *E.coli* in aquatic environments.

Figure 4.13 *Escherichia coli* survival in untreated lake water in the presence of natural microbial flora at temperatures 4 °C, 15°C and 37°C.

Figure 4.14 *Escherichia coli* survival in filtered-autoclaved lake water in the absence of natural microbial flora at temperatures 4°C, 15°C, 25°C, 30°C and 37°C.

E.coli was grown in nutrient broth overnight, harvested, washed and resuspended in sterile lake water. 100 ml aliquots of lake water were inoculated to give an initial density of between 10^6 and 10^7 cfu/ml. Flasks were incubated in the dark without shaking at the stated temperatures. Viable count were determined by surface spread plates on nutrient agar after incubation at 37°C for 24 h.

Fig.4.13

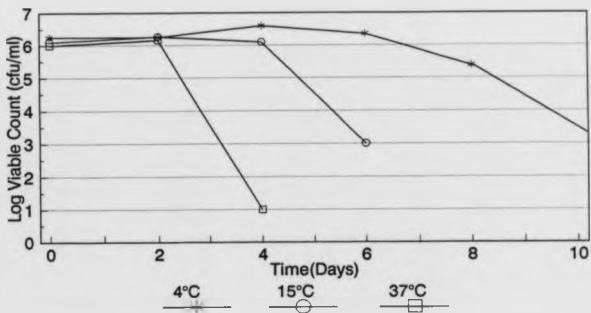


Fig.4.14

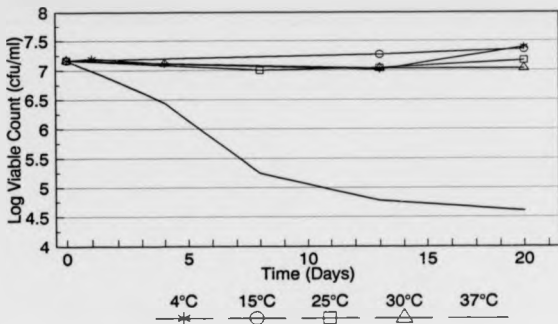


Figure 4.15 The effects of eukaryotic inhibitors on the survival of *Escherichia coli* in untreated lake water in the presence of indigenous microbial flora at temperatures 15°C and 30°C.

E.coli was grown overnight in nutrient broth, harvested, washed and resuspended in sterile lake water. 100 ml microcosms were inoculated with approximately 10^6 cfu/ml and incubated in the dark without shaking at 15°C or 30°C. The untreated lake water was amended with either 300 µg/ml cycloheximide or 250 µg/ml nystatin. Viable counts were determined by surface spread plates incubated for 24 h at 37°C.

Fig.4.15

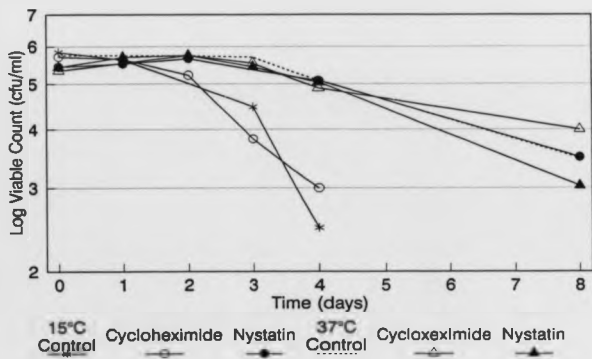


Figure 4.16 The survival of *Escherichia coli* in variously filtered lake water at 30°C.

Figure 4.17 The survival of *Escherichia coli* in variously filtered lake water at 15°C.

E. coli was grown in nutrient broth overnight, harvested, washed and resuspended in sterile lake water. 100 ml microcosms were prepared with lake water subjected to different filtration regimes, as follows:

Control untreated lake water

Whatman-filtered lake water (WF)

0.45 μm pore size Millipore-filtered lake water

0.2 μm pore size Millipore-filtered lake water

Filtered-autoclaved lake water

Each flask was inoculated to give an initial population of approximately 10^6 cfu/ml. The flasks were incubated in the dark without shaking at 15°C or 30°C. Viable counts were determined using surface spread plates incubated at 37°C overnight.

Fig.4.16

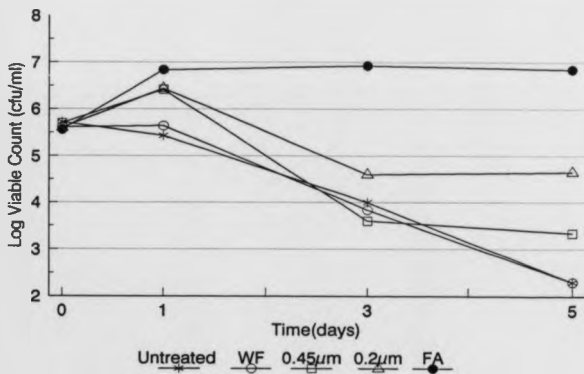


Fig.4.17

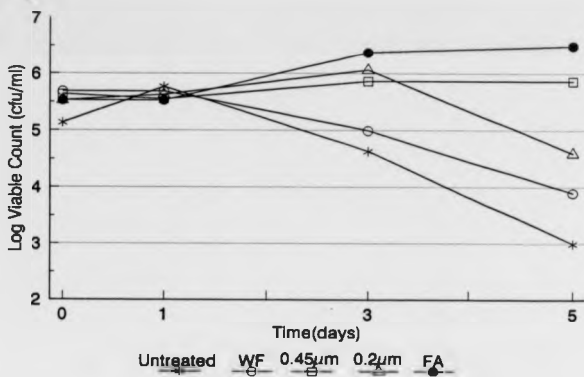
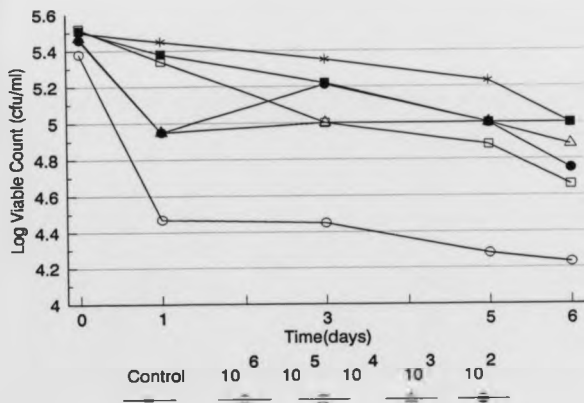


Figure 4.18 The effect of bacteriophage (K2) on the survival of *Escherichia coli* in filtered-autoclaved lake water.

E.coli was grown in nutrient broth overnight, harvested, washed and resuspended in sterile lake water. Bacteriophage K2 was diluted down from a stock culture and different titres of bacteriophages were added to the filtered-autoclaved lake water inoculated with *E.coli*. Viable counts were determined using surface spread plates incubated at 37°C overnight. The flasks were incubated at 30°C in the dark without shaking.

Fig. 4.18



4.3 Discussion

E.coli is the organism most commonly used as an indicator of faecal pollution. The presence of *E.coli* implies that other pathogens could be present in aquatic environment. Amongst the environmental parameters that commonly influence the survival of *E.coli* and other bacterial species in aquatic environments the concentration of essential nutrients is of particular importance. There is competition amongst bacteria to scavenge readily metabolizable nutrient sources in order that they may grow and survive. In the survival experiments reported in this chapter different concentrations of carbohydrates, amino acids, carboxylic acids and nitrogen sources were used as amendments to investigate the survival of *E.coli* in untreated and filtered-autoclaved lake water. The hypothesis being tested was that if a particular nutrient was limiting and leading to the decline in numbers of *E.coli* in lake water then the amendment of lake water with a selection of nutrients should allow longer survival than in the controls. The experiment were also conducted at different temperatures; mainly 15°C close to the optimum growth temperature for the natural microflora and 30°C close to the optimum growth temperature for this strain of *E.coli*.

The addition of range of carbohydrates to filtered-autoclaved lake water and untreated lake water did not produce any important differences in the survival of *E.coli* at 15°C or 30°C. However *E.coli* could grow on these carbohydrates in lake water increasing their cell numbers by up to 7 times higher than the control. Lim and Flint (1989) had also shown similar results with other similarly amended water samples. In their experiments the survival of *E.coli* was not increased at 30°C, 15°C or 4°C when carbohydrates were added to the lake water. It is possible that the carbohydrates are not taken up by *E.coli* at the lower temperatures as efficiently as by the natural microbial flora. It is shown in chapter 5 the respiration rate of *E.coli* was initially increased although

there was no increase in viable count with the addition of carbon sources to filtered-autoclaved lake water at 37°C then showed a gradual decline. This suggests that *E. coli* could use carbon sources for respiration but not any increase in bacterial numbers. In aquatic environments it is unclear how *E. coli* could compete successfully for carbohydrates with the natural microbial flora. Shetata and Marr (1971) showed that *E. coli* could grow in mineral salts medium with glucose as the carbon source at the concentration of the glucose found in most aquatic environments. This indicates that *E. coli* could take up glucose at the low concentrations found in natural environments but it is nevertheless unlikely that *E. coli* could compete with the natural microflora for glucose at its natural concentration. Some carbohydrates are transported without the need for energy utilisation, for example melibiose (West and Mitchell, 1972) and lactose (Neidhardt *et al.*, 1991). It may be speculated that this could give *E. coli* a competitive advantage was now used for growth. However these diffusion uptake pathways only function if concentrations are high. Therefore they are unlikely to function in the aquatic environment where concentrations are low. If the carbohydrates accumulate intracellularly and cannot be utilised for the generation of energy or new biomass because of the limitation of other essential nutrients then cells may be killed through this accumulation (Postgate, 1961). This is the phenomenon of substrate-accelerated death. However in the experiments reported here this accelerated death was not seen at any of the temperatures studied or with any of the carbohydrates used as amendments suggesting therefore that the carbohydrates are not being taken up to any appreciable degree.

Another group of carbon sources were used in these survival studies. The results showed that some carboxylic acids clearly enhanced the survival of *E. coli* in untreated lake water at 15°C but not at 30°C. The viable count also increased by up to 1 log within 72 h at 15°C and 30°C in filtered-autoclaved

lake water amended with a number of carboxylic acids. This suggests that either these intermediaries in metabolic pathways can be utilised more efficiently by stressed bacteria or they are taken up more efficiently from the environment. Many organic acids are important metabolites not only as a source of carbon and energy but also vital intermediates for glucogenesis and anaplerosis (Sanwal, 1970). Kay (1978) suggested that carboxylic acids were good substrate for the growth of *E. coli* because these substrates could be transported and utilised very rapidly. Brown (1972) reported that *E. coli* could grow very well on lactate as a carbon source. These were laboratory experiments in mineral salt medium. Here survival was enhanced in untreated lake water after the addition of carboxylic acids. This could have been because *E. coli* could compete successfully with the natural microbial flora for these substrates. It is also possible that these acidic end products of metabolism has an adverse effect on the natural microbial flora. If the effect had not been direct effect on *E. coli* then similar results would have been expected between the control and the filtered-autoclaved lake water amended by the addition of carboxylic acids. However survival times were increased in filtered-autoclaved lake water because *E. coli* could grow on carboxylic acids used as amendments. Pyruvate has been shown to be actively transported into membrane vesicles of *E. coli* but not of a *Pseudomonas* spp (Matin and Konings, 1973). The experiments reported here suggest that propionate and acetate had most impact on the survival of *E. coli* in untreated lake water. Both these carboxylic acids taken up by the same uptake system (Kay, 1972). Carboxylic acids as end-products or intermediates of carbohydrate metabolism seem to have a more important role in *E. coli* survival than carbohydrates themselves. This may ultimately prove to be a reflection of the stability of their uptake and utilisation mechanisms under starvation conditions.

Amino acids have noticable effects on the survival of *E. coli* in the absence of the natural microbial flora in lake water (Lim and Flint, 1989).

Hepel (1977) showed that amino acid uptake in bacteria involve periplasmic binding proteins and with the presence of other amino acids in the environment (Quay and Oxender, 1976), concentration of sodium ions (Tsuchia *et al.*, 1977), membrane potential and pH gradients (Booth and Hamilton, 1980), the intracellular amino acid pool level (Britten and McClure, 1962a). It is not unusual for amino acids and the peptides resulting from the breakdown products of proteins to produce different growth responses in *E. coli*. The growth of *E. coli* is strictly regulated by the amino acids pool level which in turn controls the amounts and activities of enzymes involved in the biosynthesis and degradation of amino acids (Payne, 1980). Control of nitrogen limitation can produced derepressed synthesis of low affinity transport systems for certain amino acids which presumably enhances utilisation free amino acids (Payne, 1980). Many of the amino acid transport systems are repressed by metabolically related derivatives for example the transport of leucine and isoleucine (Quay and Oxender, 1976), arginine, lysine and ornithine (Celis *et al.*, 1973) and aromatic amino acids (Whipp and Pittart, 1977). One additional possibility is that particular amino acids which cannot be used as a single carbon and energy source could be cocometabolized in the presence of other amino acids (Dalton and Stirling, 1982). The effects of amendments with amino acids on the survival of *E. coli* in lake water are likely to be complex because of the above arguments which illustrate the many different factors which affect the utilisation of amino acids from the environment. The results presented here showed that amino acids did not have a remarkable effect on *E. coli* survival in the presence of natural microbial flora. However amino acids were taken up and allowed the *E. coli* cells to grow in filtered-autoclaved lake water. Growth was usually followed by a gradual decline in viability. In aquatic environments ammonium ions and amino acids can be taken up by active transport systems coupled to protein synthesis (Stevenson and Silver, 1977; Kirchmann and Hodson, 1984) or by passive diffusion. In nutrient-limited oligotrophic environments amino acid

uptake was more efficient for natural isolates from oligotrophic environments than in copiotrophic organisms such as *E.coli*. Hence *E.coli* would not be able to compete for these essential nutrients at natural concentration (Vaccaro, 1965). It is therefore not a surprise that amino acids had little effect on the survival of *E.coli* in untreated lake water.

The addition of nitrogen sources particularly casein and ammonium ions had the greatest effect on the survival of *E.coli*. Increasing concentration of nitrogen sources led to longer survival of bacteria in untreated lake water samples especially at 15°C. Ammonia is actively taken up from the environment by an energy dependent and the energy independent process (Neidhardt *et al.*, 1991). Ammonia assimilation is very important as 14% of dry mass of bacteria is nitrogen. Ammonia may be produced intracellularly by the breakdown of urea, arginine, histidine, glutamate and the other nitrogen-containing compounds. Stevenson and Silver (1977) reported that three strains of *E.coli* concentrated ammonium ions intracellularly to concentrations over 100 times that in the external environment. One can argue that in the natural environment *E.coli* is likely to have a high affinity uptake system. Thus this could allow *E.coli* to survive longer even under unfavourable conditions if ammonium ions could still be taken up for instance under starvation conditions at temperatures of 15°C or less. High concentration of ammonia could give *E.coli* an advantage over the indigenous microflora in untreated lake water thus increasing the survival times of *E.coli*. The indigenous microflora is more likely to have adapted to survive under conditions of low ammonium concentrations in the aquatic environments where ammonia concentrations rarely exceed 12 µg/l except where water is polluted by sewage effluent (Vaccaro, 1965). As a result higher concentrations of ammonia ion as used here as an amendment might be toxic to the natural microbial flora although there was no evidence for this. Although there is considerable diversity in the way bacteria obtain ammonium

ion they incorporate it into organic constituents of the cell by only two pathways, ammonia can be assimilated directly into glutamate via a reaction catalysed by L-glutamate dehydrogenase (GDH) (Brown *et al.*, 1974) or indirectly by a cycle of reactions (GS-GOGAT) (Tempest, 1973) catalysed by glutamine synthetase. GS has a considerable higher affinity for ammonia ions than does GDH. GS catalysed reactions utilise ATP, the GDH reaction does not. Some bacteria possess one of these two pathways, others, such as *E.coli*, possess both. GS synthesis is repressed in organisms growing in good nitrogen sources such as glutamine or at ammonia concentrations in which case ammonia assimilation proceeds via GDH and ammonia can substitute for glutamine in many biosynthetic reactions.

It was shown in the present experiments that less than 50 mg-N/l of ammonium sulphate did not cause any changes in the survival of *E.coli* in untreated lake water but increased survival was seen at higher concentrations of ammonium sulphate. Similar results were obtained with ammonium nitrate but not with potassium nitrate or sodium nitrate. This suggests that it was the ammonium ion moiety which was important. It is possible that high ammonium concentration were toxic to other competitors or to predators but this was considered unlikely in previous studies (Lim and Flint, 1989). *E.coli* must be capable of taking up ammonium ions from the environment under the starvation conditions used here. The increased survival must then be due to channelling of these ammonium ions into protein synthesis or the change in the internal pH which would result from accumulation of ammonium ions changing metabolic process to enhance the survival of bacterial cells. This increase survival of *E.coli* in the presence of ammonium ion is of importance because although ammonia and *E.coli* are likely to be introduced from the same source i.e. sewage effluent but ammonia could also be introduced in farm slurries and fertiliser wastes.

Most *E. coli* are introduced into water in sewage effluents, hence the survival of *E. coli* in water amended with sewage effluent is of importance. In this present study *E. coli* survival was increased by the addition of a synthetic sewage to the lake water and survival was temperature dependent. Although *E. coli* could not grow well in untreated lake water, longer survival occurred than in amended controls. However, *E. coli* grew efficiently in filtered-autoclaved lake water at 15°C. This is likely to be due to the absence of competition for nutrients in the filtered-autoclaved lake water. Flint (1987) and Lim and Flint (1989) showed that temperature was an important factor in the survival of *E. coli*. Here temperature is important for the utilisation of the sewage as a nutrient source by *E. coli*. It is possible that enhanced survival in the sewage-amended water is simply reflection of the increased ammonium ion concentration of the water.

The results presented here imply that it is competition for nutrients which is the main factor for the decline on *E. coli* numbers under starvation conditions. The role which predation plays in the elimination of bacteria was investigated in water samples subjected to differential filtration procedures. Alexander (1989) have suggested that it is only the slow-growing bacteria which are eliminated by protozoan feeding and any bacterium which is going to colonise an aquatic environment or survive for any appreciable time has to be able to grow at a rate in excess of predation. Mitchell and Yankofsky (1969) reported that population of *Vexillifera telmhalassa* increased as the number of *E. coli* declined in river water microcosms. Many other researchers have suggested that predation by protozoa could be the main factor leading to the decline in the numbers of *E. coli* in lake water samples (McCambridge and McMeekin, 1981; Barcina *et al.*, 1986; Rhodes and Kator, 1989; Garcia-Lara *et al.*, 1992). Here the survival of *E. coli* in nonsterile lake water was examined. The results suggest that in this situation protozoan predation and bacteriophage were of minor importance in the

disappearance of *E.coli* seen in the untreated lake water in the presence of natural microflora. Using metabolic inhibitors to inhibit the function of protozoa did not lead to dramatic changes in survival although slightly longer survival was noted. These results suggested that elimination of *E.coli* by eukaryotic cells was not the major reason for their disappearance. The survival of *E.coli* was increased at both 15°C and 30°C by filtration of the water through 0.45 µm and 0.2 µm Millipore filters. This could be due to complete elimination of predators or to the elimination of the bacterial competitors. Microscopic observation of Whatman-filtered samples revealed no protozoa in these samples therefore we believe that the differences in survival seen between the Whatman- and Millipore filtered water are due to the elimination or removal of the bacterial component of the microflora.

Millipore-filtered water will still contain bacteriophage particles which could be important in the reduction of *E.coli* numbers seen in these samples after a period of incubation at 15°C and 30°C. A reduction was rarely seen in Millipore-filtered samples kept at 4°C. A similar reduction in the numbers of *E.coli* surviving in river water had been reported by Flint (1987). Incubation of *E.coli* in lake water samples to which bacteriophage had been added revealed that titres of less than 10^3 particles per ml were ineffective in reducing the numbers of *E.coli*. The phage used had been isolated by enrichment techniques from lake water samples. It is not known what the natural concentration of this or comparable bacteriophage able to produce plaques on *E.coli* but it is presumed that natural titres are extremely low. In natural environments the probability of contact occurring between a bacterial cell and a phage particle is considered as being very remote. Hence we conclude that bacteriophage only play a minor role in the disappearance of *E.coli* compared to the role of the bacterial microflora.

In conclusion the survival of *E.coli* was longest at all temperatures in samples from which all the bacteria, protozoa and bacteriophage had been eliminated, i.e. sterile lake water. The complete elimination of the biotic component of lake water is essential if *E.coli* is to survive for any length of time. The addition of ammonium ions to the water however can lead to survival even in untreated lake water.

**CHAPTER V. The effect of starvation on the Respiratory activity of
Escherichia coli.**

5.1 Introduction

One of the main purposes of respiration in living organisms is the production of energy to drive the growth processes necessary for their survival in the environment. However the respiration rate of bacterial cells must be appropriate to the conditions of growth in an environment exposed to radical and rapid alterations. Different factors affect the respiration rate of bacteria, for example substrate concentration (Harrison, 1976), temperature (Harrison, 1976; Olanczuk-Neyman and Vosjan, 1977; Trevors, 1984;), starvation (Boyle and Ensign, 1970; Novitsky and Morita, 1978; Dawson *et al.*, 1981; Kjelleberg *et al.*, 1982; Kurath and Morita, 1983; Smigielsky *et al.*, 1989, 1990;), pH (Harrison and Loveless, 1971), dissolved oxygen tension (Harrison, 1976) and some inhibitors (Kjelleberg *et al.*, 1983). It has been suggested that the ability of bacteria to survive during starvation is related to their ability to reduce their rate of endogenous respiration (Dawes, 1976). Kurath and Morita (1983) have reported that the majority of cells lost the ability to reproduce and respire during the course of starvation. They have also suggested that in oligotrophic waters it would be an advantage for cells to maintain their energy (ATP) reserves. This would ensure that active transport mechanisms are ready when they are required for the utilisation of nutrient sources for energy. Boyle and Ensign (1970) showed that the endogenous respiration of *Arthrobacter crystallopoietes* decreased 80-fold during the first day of starvation but then remained constant for a 24 d starvation period. Similar results were demonstrated by Dawson *et al.* (1981), Novitsky and Morita (1978) and Smigielsky *et al.* (1989) that during starvation, marine vibrios reduced their rate of respiration very significantly. Furthermore Martinez *et al.* (1989) suggested that the significant decline in electron transport system (ETS) activity in *E. coli* indicated that the inactivity of the metabolic enzymes involved in the ETS reactions was associated with culturability and cell lysis. The decline in the respiration rate was inversely related to cell size reduction under starvation stress. Smigielsky *et al.* (1989)

suggested that there might be a link between cell size reduction and low respiration rate in *V. fluvialis*.

The rate of respiration in bacterial cells is not only affected by starvation but also by excess concentrations of nutrients. The respiration rate of organisms growing under growth restricted conditions in continuous culture will generally be lower than that of the same organisms removed from the culture and supplied with excess of substrate and oxygen (Harrison, 1976). Harrison (1976) has also stated that respiration rate depends on the availability of the oxidizable substrate to the cell and is a function of growth rate. This implies that the response of growth rate to substrate concentration would also apply to respiration rate. Harrison and Maitra (1969) found that the rate of respiration was dependent not only on the concentration of nutrient sources but also their chemical nature. For example the fastest respiration rate occurred after the addition of excess glucose to the medium. Succinate, fumarate and acetate were each respired at different but lower rates. In addition, some amino acids such as aspartic acid increased respiration rate while some others such as leucine and lysine lead to a decline (Walker and Colwell, 1976). The increase in the respiration rate must affect a severe imbalance in the cell energy charge (Atkinson, 1968) and might indirectly affect the viability of the bacteria. This could be an explanation for the phenomenon of substrate-accelerated death (Postgate, 1961).

Another important factor affecting respiration rate is the alteration of the temperature in an environment. A sudden change in temperature might influence an immediate change in the maximum growth rate and therefore a change in the potential QO_2 (specific oxygen uptake rate). The increase in growth rate requires a higher rate of synthesis of RNA and some other necessary molecules whose concentrations may limit growth and respiration rate at the lower temperatures (Harrison, 1976). Trevors (1984) reported that usually minimum loss of potential for respiration activity took place at 4°C. Incubation temperature has a

significant effect on respiration rate for instance 37°C provides increased activity compare to lower temperatures.

Respiration rate of bacteria is also a function of substrate uptake rate and is therefore related to substrate concentration and temperature. However, as respiration rate is also affected by other environmental parameters, a simple method for the control of respiration by substrate supply alone, would not allow for balanced energy metabolism and growth in a substrate-limited environment (Harrison, 1976).

A major aim of recent ecological research has been to develop and improve upon methods for assessing respiring cells in pure culture and aquatic water samples. Different methods have been used for the estimation of bacterial metabolic activity *in situ* in aquatic environments. Generally ETS activity and some of the respiratory enzyme activities, such as succinate and NADH oxidase and dehydrogenase activity, have been used in order to evaluate the contribution of microbes to the cycle of material and energy in aquatic environment. In addition, ETS enzymes or dehydrogenases are responsible for taking hydrogen from a substrate and passing it along a set of carriers to free oxygen. Tetrazolium salts (INT) are one of the alternative approach as to the measurement of the activity of the electron transport system. In this method triphenyltetrazolium chloride or other tetrazolium salts, which are artificial electron acceptors, are reduced to coloured formazan which can be extracted and quantified (Trevors, 1984; Stolp, 1988). This has been employed with some success for the analysis of respiratory activity of plankton (Hobbie *et al.*, 1972), and benthos samples (Zimmerman, 1975). Kenner and Ahmed (1975a,b) have suggested that ETS activity might provide a method for calculating the oxygen uptake potential of a microbial population if a suitable ratio between formazan formation and uptake exists. Martinez *et al.* (1989) found that the ETS activity of *E. coli* declined in seawater parallel to the decline in the plate count. Previous studies have shown that a large proportion of the total metabolic activity in

aquatic bacteria can be linked to ETS activity (Haddock and Jones, 1977; Tabor and Neihof, 1982). On the other hand, ETS activity has been used as an estimation of the number of active respiring cells in aquatic environments (Zimmerman *et al.*, 1978; Trevors, 1984). This might be useful to detect viable but non-culturable bacteria under starvation and also other stress conditions.

Succinate dehydrogenase activity and NADH dehydrogenase activity are the main immediate electron donors to the respiratory chain. The reduced form of nicotinamide nucleotides are not only a useful indicator of respiratory control mechanisms but also quite conveniently monitored in living cells (Harrison, 1976). Smigielsky *et al.* (1989) showed that the viability of *V. fluvialis* began to decline after 2-3 days of starvation. During this time, the respiratory potential of bacteria decreased by 4 to 5-fold, as a result of a decrease in succinate and NADH dehydrogenase activity. It was found that dehydrogenase activity in ETS would be inhibited by low cyanide concentrations. In the presence of uncouplers, sensitive cells lose their ability to couple energy to active transport. The action of uncouplers in dissociating oxidation from phosphorylation so that the rate of respiration is increased but without the concomitant synthesis of ATP (Hamilton, 1975). CCCP (carbonylcyanide-m-chlorophenylhydrazone) collapses both pH gradients and the membrane potential components of the total proton motive force (Hamilton, 1975). Smigielsky *et al.* (1990) found that the addition of the protonophore (CCCP) or sodium azide, which both affect the respiratory chain, prevented cell size reduction during periods of nutrient limitation.

In this chapter, the respiratory activity of *E. coli* under starvation and different nutrient conditions was measured in order to investigate a relation between respiration and survival of *E. coli* in lake water microcosms. Succinate and NADH dehydrogenase activity were also measured under starvation stress in the presence or absence of respiratory inhibitors. Under starvation stress cell size measurement, total protein measurement and viable count were carried out

to find out whether these enzymes have a function in the formation of viable but non-culturable cells of *E.coli*.

5.2 Results

5.2.1 Electron transport system (ETS) activity of *Escherichia coli* under different conditions in filtered-autoclaved lake water.

5.2.1.1 The effect of changing cell numbers and incubation time on ETS activity of *Escherichia coli*.

In this experiment, the method was optimised by determining the accumulation of formazan deposits in *E.coli* cells by altering the inoculum size and the length of the incubation period. Samples were collected from a nutrient broth culture at stationary phase and the formation of formazan measured after the addition of INT substrate to the medium at 37°C. The reduction of INT was increased by cell increasing the initial inoculum size from 10^6 cfu/ml to 10^7 cfu/ml and doubled again in case of changing initial inoculum size to 1×10^8 cfu/ml (Fig. 5.1). Increasing the number of cells in the medium resulted in coloration in the sample because of the high proportion of INT chloride reduced to INT formazan in the sample. In addition when *E.coli* cells were incubated at 37°C after the addition of INT and ETS activity was examined at 20 minute intervals up to 80 minutes INT reduction and formazan deposition or ETS activity were increased in *E.coli* in line with the incubation time (Fig. 5.2). As ETS activity is a reflection of the total cell mass in the sample, nutrient sources were added to filtered-autoclaved lake water in order to compare viable count and ETS activity. Again this showed that ETS activity was related to total number of bacteria. As can be seen in one of the representative graphs in Figure 5.3a and Figure 5.3b the increase in cell number after 4-6 days of incubation in filtered-autoclaved lake water amended with 4 mg/l lactose was paralleled by an increase in the ETS activity. After 8 days of incubation as the viable count declined, ETS activity also declined. These results suggest that ETS activity may reflect the number of active cells in the sample.

Figure 5.1 The effect of initial inoculum size on electron transport system activity of *Escherichia coli*.

E. coli was grown in nutrient broth overnight at 30°C then culture diluted down with sterile distilled water to 10^{-1} , 10^{-2} and 10^{-3} . 10 ml aliquots were removed from these dilution including approximately 10^6 , 10^7 or 10^8 cfu/ml and transferred to 20 ml of sterile universals. 1 ml 0.2% of aqueous INT solution was added and universals incubated in the dark at 37°C for 20 minutes. Activity was stopped by the addition of 0.1 ml formaldehyde to the samples. Reduced INT was extracted in 1 ml methanol at 70°C and the cell debris was removed by centrifugation. ETS activity was assessed spectrophotometrically at 490 nm. Activity is expressed as change in absorbance at 490 nm/20 min.

Figure 5.2 Changes in electron transport system activity (ETS) of *Escherichia coli* according to the length of incubation at 37°C.

E. coli was grown in nutrient broth overnight at 30°C then culture diluted down to 10^{-3} . 10 ml of aliquots were set up in the 20 ml universal including approximately 10^6 cfu/ml of initial viable count. 1 ml 0.2% aqueous INT solution was added and the universals incubated in the dark at 37°C for 20, 40, 60 and 80 min. For each interval the activity was stopped by the addition of 0.1 ml formaldehyde. Reduced INT was extracted in 1 ml methanol at 70°C and the cell debris was removed by centrifugation. ETS activity assay was assessed spectrophotometrically at 490 nm. Activity was expressed as change in absorbance at 490 nm.

Fig.5.1

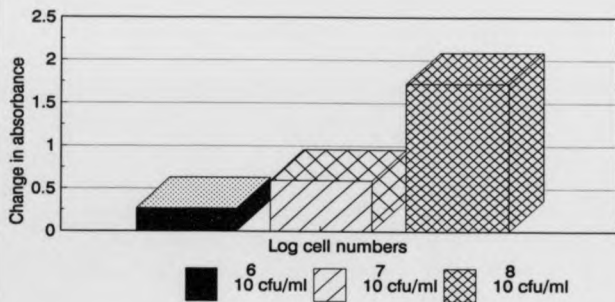
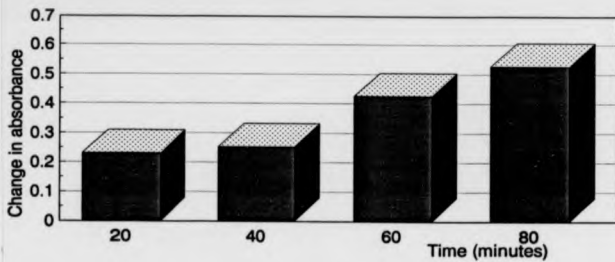


Fig.5.2



5.2.1.2 ETS activity of *Escherichia coli* under starvation stress

It has been reported that starvation leads to a slow down in the metabolic activity of the bacterial cell especially in the respiration rate. In these experiments, ETS activity was measured in *E.coli* starved in filtered-autoclaved lake water at different temperatures, in order to explore the link between survival of *E.coli* and ETS activity as a measure of respiration rate under starvation condition. As regard to results at 0 day rate of ETS activity occurred at a higher rate in *E.coli* cells incubated at 30°C than 4°C and 15°C (Fig. 5.4a). After a day while ETS activity began to decline sharply at 30°C, a gradual increase in activity occurred in cells incubated at 4°C and 15°C. While loss of viability was 398.4-folds, there was a 5.96-fold in case of ETS activity of *E.coli* cells at 30°C after 6 days of incubation (Fig. 5.4a and 5.4b). On the other hand ETS activity gradually increased for the first 6 days of the starvation period at 4°C and 15°C. The number of cells remained constant for up to 12 days of incubation at these temperature (Fig 5.4b). The minimum inoculum size to measure ETS activity in *E.coli* in lake water have been shown to be approximately 10^5 cfu/ml. Here, although the viable count decreased to 3×10^4 cfu/ml after 6 days of incubation, ETS activity was still measurable (Fig. 5.4a). This suggests that although the majority of the cells might lose the ability to form colonies (i.e. become non-culturable), they are capable of ETS activity and hence the ability to respire.

ETS activity in *E.coli* was also determined after the addition of nutrient broth to cells starved in filtered-autoclaved lake water for 2, 4, 7 and 16 days at 15°C and 30°C. The aim was to investigate the response of starved cells to nutrient addition with regard to the period of starvation. The ETS activity was performed three times on each to the filtered-autoclaved lake water. At 30°C (Fig. 5.5a), the specific activity after 2 and 6 h was increased with the increased starvation period but there was a little change in the specific activity 24 h after the addition of nutrient broth. This suggested that the cells could respond to the

addition of nutrient broth very quickly even after a long period of starvation. The increased specific activity with the increased period of starvation may simply be a reflection of the decreasing viable count. The specific activity of the cells was always lower in those starved at 15°C (Fig. 5.5a). Again this was probably a reflection of the more stable viable count at this lower temperature. The response of the cells to the nutrient broth addition was also slower with a higher proportional of the specific activity remaining 24 h after the addition of nutrient broth. At both temperatures these results suggest that the ETS activity of *E. coli* is unimpaired even by starvation periods of up to 16 d in the absence of exogenous nutrient sources and that the ability of the cells to respond to the addition of a nutrient source (in this case nutrient broth) is unimpaired with an increasing starvation period.

5.2.1.3 The effects of nutrient amendments on ETS activity of *Escherichia coli*.

The effects of carbon sources on *E. coli* survival have been reported in Chapter 4. Some carbon sources led to an increase in the number of *E. coli* cells but later led to an accelerated rate of decline in cell number. Some of these carbon sources have been examined in terms of the changes in ETS activity or respiration rate. It was observed here that some of the carbon sources such as sucrose, fructose and succinate caused an increase in ETS activity in filtered-autoclaved lake water both at 15°C and 30°C (Fig. 5.6a; Fig. 5.6b and Fig. 5.6c). The noticeable increases in ETS activity occurred with the addition of succinate, fructose and sucrose to filtered-autoclaved lake water at 30°C. This is not a reflection of an increased number of cells in the sample because the rate of activity was calculated as specific activity of the cells. In general, the alteration in ETS activity in *E. coli* took place within a range 1.26 to 6.98-fold at 4°C, 1.25 to 5.12 at 15°C and 0.49 to 18.0 at 30°C after the addition of nutrient sources to filtered-autoclaved lake water (Table 5.1). The increase in specific

activity in filtered-autoclaved lake water after the addition of nutrient sources at 4°C was a surprising result because low metabolic activity was expected to be reflected in a low respiration rate. One of the representative graphs is presented in Figure 5.7 and shows that increasing the concentration of lactose led to an increase in ETS activity proportional to concentration although with the addition of 6 mg-C/l activity started to decline after 3 days. The addition of 4 mg-C/l of lactose resulted in the highest ETS activity in *E.coli* cells in filtered-autoclaved lake water after 10 days at 15°C. Other carbon sources showed similar concentration dependent responses.

The addition of increasing concentrations of amino acids also produced a proportional increase in ETS activity of *E.coli*. As shown in representative graphs in Figure 5.8 and 5.9, leucine and proline had a concentration dependent effect on ETS activity at both 15°C and at 30°C.

5.2.2 The activity of respiratory enzymes in *Escherichia coli* in lake water under starvation stress.

In these experiments succinate and NADH dehydrogenase activity of *E.coli* was detected under starvation conditions at different temperatures. It was shown in chapter 3 that cells remained viable but non-culturable in filtered-autoclaved lake water at 30°C. Here succinate and NADH dehydrogenase activity were measured in *E.coli* to understand the relationship between these dehydrogenases and starvation survival of *E.coli*.

5.2.2.1 Succinate dehydrogenase and NADH dehydrogenase activity of *Escherichia coli* under starvation condition at different temperatures.

It is often stated that the respiration rate of bacteria including *E.coli* is reduced under starvation stress. However, activity of succinate and NADH dehydrogenase has been detected in dormant cells in soil and aquatic environments. Figure 5.10 and 5.11 shows that succinate dehydrogenase activity

Figure 5.3 The comparison of viable count and ETS activity of *Escherichia coli* in filtered-autoclaved lake water at 30°C.

E. coli was grown in nutrient broth overnight at 30°C, harvested, washed and resuspended in sterile lake water. 100 ml lake water microcosm were amended with a final concentration of 4 mg-C/l lactose and inoculated to give an initial viable count of approximately 10^7 cfu/ml. Viable counts were determined on surface spread plates after overnight incubation at 30°C (Fig. 5.3a).

For the measurement of ETS activity, 10 ml of samples were taken into sterile universals and 1 ml of 0.2% aqueous INT solution added. The universals were incubated in the dark at 37°C for 60 min. Activity was stopped by the addition of 0.1 ml formaldehyde. Reduced INT was extracted in 1 ml methanol at 70°C and cell debris was removed by centrifugation. ETS activity assay was assessed spectrophotometrically at 490 nm. Activity is expressed as change in absorbance at 490 nm, per h. (Fig. 5.3b).

Fig.5.3a

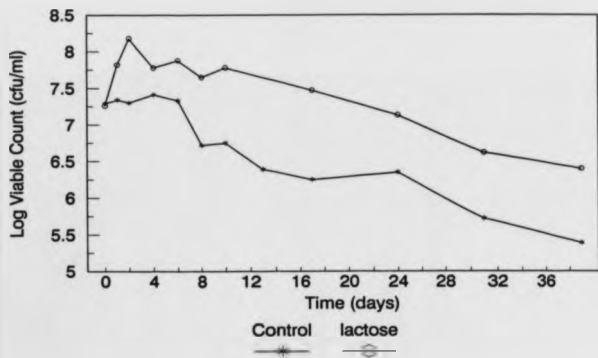


Fig.5.3b

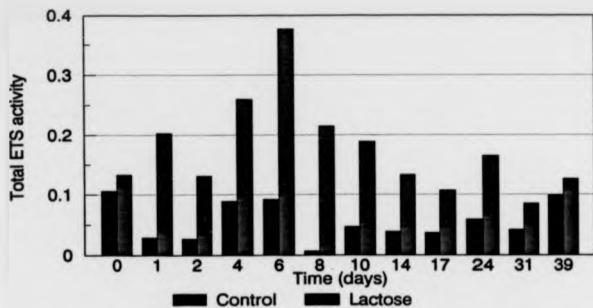


Figure 5.4 Electron transport system activity of *Escherichia coli* under starvation stress at different temperatures.

E. coli was grown in nutrient broth overnight at 30°C, harvested, washed and resuspended in sterile lake water. 100 ml filtered-autoclaved lake water microcosms were inoculated to give an initial viable count of approximately 10^7 cfu/ml. Viable counts were determined on surface spread plates after overnight incubation at 37°C (Fig. 5.4). 10 ml of aliquots were taken into 20 ml sterile universal and 1 ml of 0.2% aqueous INT solution added. The universals were incubated in the dark at 4°C, 15°C and 30°C for 24 h. Activity was stopped by the addition of 0.1 ml formaldehyde. Reduced INT was extracted in 1 ml methanol at 70°C until pellet was completely bleached and then cell debris was removed by centrifugation. ETS activity assay was assessed spectrophotometrically at 490 nm. Activity is expressed as change in absorbance at 490 nm per h.

Fig.5.4a

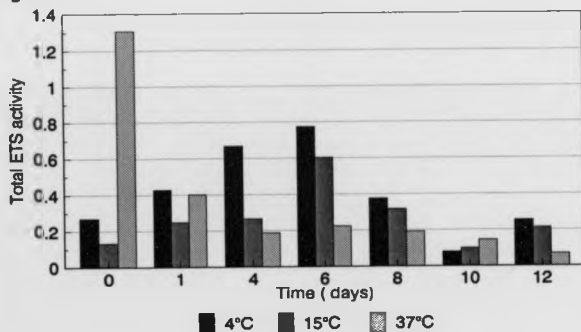


Fig.5.4b

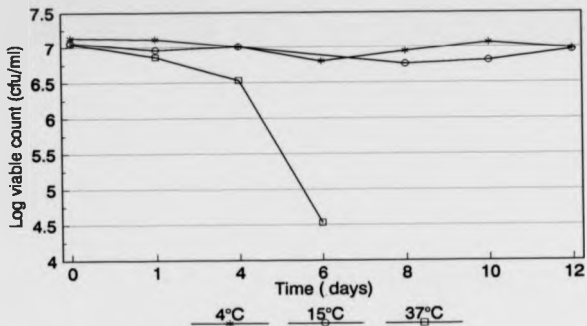


Figure 5.5 Electron transport system activity after the addition of nutrient broth to lake water starvation medium of *Escherichia coli* at 30°C (Fig. 5.5a) or 15°C (Fig. 5.5b).

E. coli was grown in nutrient broth overnight at 30°C, harvested, washed and resuspended in sterile lake water. 100 ml filtered-autoclaved lake water microcosms were inoculated to give an initial viable count of approximately 10^7 cfu/ml. Each flask was kept for different starvation period of 2, 4, 7 or 16 day. Experiments were carried out three times a day (2, 6 and 24 hours after the addition of 1 ml sterile nutrient broth to the starved lake water medium). 10 ml aliquots were added to 20 ml universals and 1 ml of 0.2% aqueous INT solution were added. The universal were incubated in the dark at 15°C or 30°C for 24 h. The activity was stopped by the addition of 0.1 ml of formaldehyde. Reduced INT was extracted in 1 ml methanol at 70°C and cell debris was removed by centrifugation. ETS activity was assessed spectrophotometrically at 490 nm. Specific activity is expressed as change in absorbance at 490 nm per 24 h.

Fig.5.5a

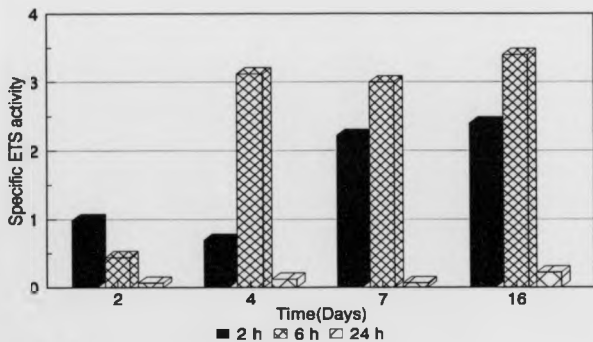


Fig.5.5b

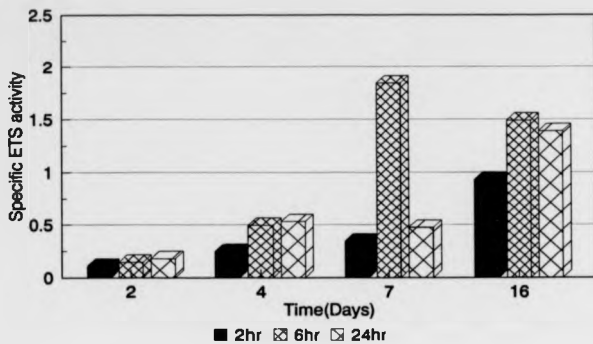


Figure 5.6 The effect of carbon sources on the electron transport system activity of *Escherichia coli* in filtered-autoclaved lake water at 15°C and 30°C.

E. coli was grown in nutrient broth overnight at 30°C, harvested, washed and resuspended in sterile lake water. 100 ml filtered-autoclaved lake water microcosms were individually amended with 4 mg-C/l sucrose (Fig. 5.6a), 4 mg-C/l D-fructose (Fig. 5.6b) and 2 mg-C/l of succinate (Fig. 5.6c) and inoculated to give an initial viable count of approximately 10^7 cfu/ml. 10 ml aliquots were removed to 20 ml sterile universal. 1 ml of 0.2% aqueous INT solution were added. The universals were incubated in the dark at 15°C or 37°C for 24 h. The activity was stopped by the addition of 0.1 ml of formaldehyde. Reduced INT was extracted in 1 ml methanol at 70°C and cell debris was removed by centrifugation. ETS activity was assayed spectrophotometrically at 490 nm. Specific activity is expressed as change in absorbance at 490 nm per 24 h.

Fig. 5.6a



Fig. 5.6b

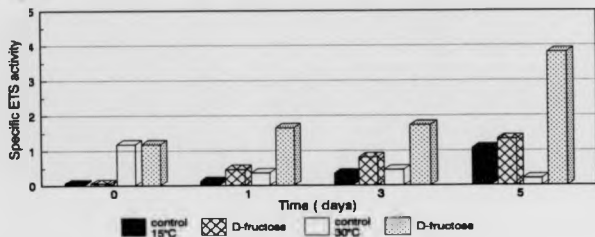


Fig. 5.6c

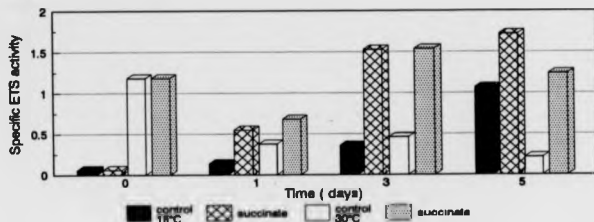


Figure 5.7 The effect of increasing concentration of lactose on electron transport system activity of *Escherichia coli* in filtered-autoclaved lake water at 15°C.

Figure 5.8 The effect of increasing concentration of leucine on electron transport system activity of *Escherichia coli* in filtered-autoclaved lake water at 15°C.

Figure 5.9 The effect of increasing concentration of L-proline on electron transport system activity of *Escherichia coli* in filtered-autoclaved lake water at 30°C.

E. coli was grown in nutrient broth overnight at 30°C, harvested, washed and resuspended in sterile lake water. 100 ml filtered-autoclaved lake water microcosms were individually amended with 2 to 6 mg-C/l lactose, 2 to 6 mg/l leucine and 2 to 6 mg/l L-proline and inoculated to give an initial viable count of approximately 10^7 cfu/ml. 10 ml of aliquots were removed to 20 ml sterile universals and 1 ml of 0.2% aqueous INT solution was added. The universals were incubated in the dark at 15°C or 30°C for 24 h. The activity was stopped by the addition of 0.1 formaldehyde. Reduced INT was extracted in 1 ml methanol at 70°C and the cell debris was removed by centrifugation. ETS activity was assayed spectrophotometrically at 490 nm. Specific activity is expressed as change in absorbance at 490 nm per 24h.

Fig.5.7

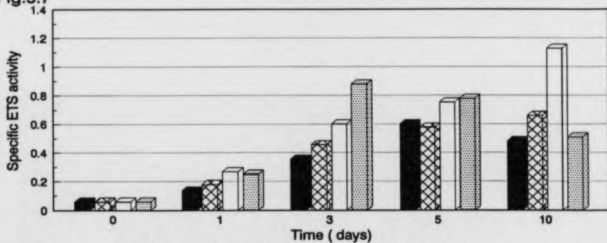


Fig.5.8

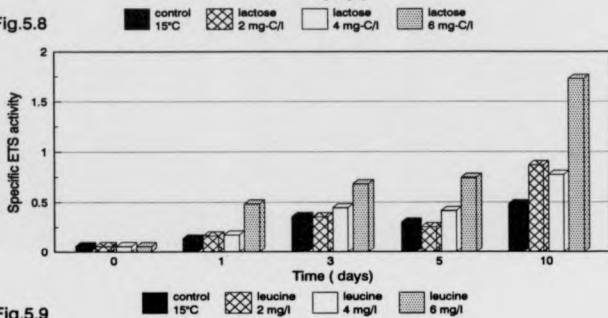


Fig.5.9

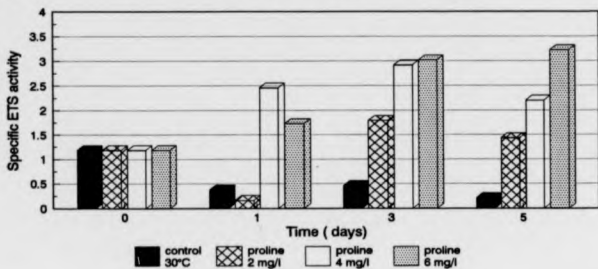


Table 5.1 The effect of different nutrient sources on electron transport system activity of *Escherichia coli* in filtered-autoclaved lake water at different temperatures.

Substrates and Concentrations (per/ml)	15°C Specific ETS activity	Ratios	30°C Specific ETS activity	Ratios	4°C Specific ETS activity	Ratios
control (none)	0.355	1.00	0.214	1.00	0.126	1.00
sucrose (4 mg)	0.781	2.20	4.4	20.5	1.410	11.10
D-fructose(4 mg)	0.91	2.56	3.8	17.75	1.880	14.90
glycerol (2 mg)	1.150	3.23	2.000	9.34	0.180	1.26
lactose (4 mg)	0.601	1.69	3.940	18.40	0.380	3.01
succinate (2 mg)	1.820	5.12	1.340	6.26	0.310	2.46
acetate (4 mg)	1.260	3.54	1.244	5.81	0.770	6.11
formate (4 mg)	0.968	2.77	0.105	0.49	0.200	1.58
casein (2 mg)	0.837	2.35	0.779	3.64	0.170	1.36
glycine (4 mg)	0.444	1.25	0.482	2.25	0.340	2.69

Specific ETS activity were measured after 3 d of incubation for 4, 15°C and 5 d for 30°C.

showed differences almost proportional to the temperature after 24 h of starvation stress. The decline in the activity of these enzymes took place for all temperatures in filtered-autoclaved lake water under nutrient deprivation. After 48 h of incubation the highest activity of succinate dehydrogenase occurred in cells starved at 4°C and lowest activity occurred in those starved at 37°C. The results presented in Chapter 3 show that the survival of *E.coli* did not alter at 4°C but the disappearance of *E.coli* was the fastest at 37°C. This results suggest that succinate dehydrogenase activity decreases as *E.coli* becomes subjected to starvation stress in filtered-autoclaved lake water.

NADH dehydrogenase activity was also detected in *E.coli* starved in filtered-autoclaved lake water at different temperatures. There were noticeable differences in NADH dehydrogenase activity in *E.coli* incubated at 4°C, 15°C, 25°C and 37°C in filtered-autoclaved lake water. Again the NADH dehydrogenase activity started to decline immediately after inoculation of the cells into lake water but was still detectable even after 13 days of incubation. The lowest activity was measured at 37°C (Fig. 5.11) but activity remained at a higher level in cells incubated at the lower temperatures in filtered-autoclaved lake water. It is possible that NADH dehydrogenase activity of *E.coli* declined in order to maintain energy reserves for a long-term starvation stress. It would therefore be more likely that activity would decline faster at the higher temperatures. The results suggest that the reason for the disappearance of *E.coli* or the appearance of a non-culturable form in lake water is not likely to be cell death but the slowing down of some metabolic mechanisms, such as succinate and NADH dehydrogenase activity. The total amount of protein in *E.coli* cells remained a little higher at 37°C than at the other incubation temperatures after 13 days of starvation (Fig. 5.12). However there was a steady decline in the protein content of the cells, although there was no measurable difference between the protein content of cells starved at 4, 15, 25, and 30°C.

In Chapter 3 it was shown that the reduction in viable count and the cell size of *E. coli* occurred at a higher rate at 37°C than at 4°C. Similar results were obtained here for ETS activity and also for the respiratory enzymes succinate and NADH dehydrogenase. There was also a possible link between viable count, the viable but non-culturable state and ETS activity or respiration rate, and incubation temperature. One of the features of bacteria becoming viable but non-culturable is cell size reduction under starvation stress. Under the same conditions cell size reduction of *E. coli* was analysed in filtered-autoclaved lake water under starvation stress and showed that the largest cell size reduction occurred in *E. coli* cells at 37°C that the cell size did not alter significantly at 4°C and little reduction at 15°C. All the above results suggest that entering viable but non-culturable state by *E. coli* may have a positive relationship with metabolic activity and respiration rate.

5.2.2.2 The effects of uncoupling agent on succinate and NADH dehydrogenase activity of *Escherichia coli* in filtered-autoclaved lake water.

Bacterial cells lose their ability to couple energy after the addition of uncouplers such as carbonyl cyanide-*m*-chlorophenylhydrazone (CCCP) and sodium azide which destroy both the membrane potential components of the total proton motive force and pH gradients (Hamilton, 1975). Figure 5.13, 5.14, 5.15 and Figure 5.16 show the effect of adding CCCP and sodium azide to filtered-autoclaved lake water on succinate dehydrogenase and NADH dehydrogenase activity over a 7 day incubation period. As is shown in Figure 5.13 final concentrations of 5 μ M and 10 μ M CCCP led to faster cell respiration compared to the control. The rate of succinate dehydrogenase activity declined to a quarter of the original level. In the same experiment showed that total protein changes was almost the same for all the samples. The viable count was also reduced by the addition of 10 μ M CCCP. The reason for that could be the high respiration rate concomitantly without ATP synthesis in the cells. In

addition CCCP addition to the filtered-autoclaved lake water did not cause any significant cell volume changes under starvation conditions.

The same concentration of CCCP did also result in a increase in NADH dehydrogenase activity compared to the control (Fig. 5.14). There was no significant changes in activity after the addition of 5 μ M or 10 μ M CCCP to filtered-autoclaved lake water. On the other hand, sodium azide did not affect activity of either succinate or NADH dehydrogenase activity. It is known that concentration of 50 μ M to 100 μ M of sodium azide can affect survival of *E.coli*. These results suggest that sodium azide effect on succinate dehydrogenase and NADH dehydrogenase enzyme activity was not significant but there is probably interaction with other metabolic processes in the cells. As a result sodium azide addition leads to decline survival of *E.coli* in filtered-autoclaved lake water.

Figure 5.10 Succinate dehydrogenase activity of *Escherichia coli* in filtered-autoclaved lake water.

E. coli was grown in nutrient broth overnight at 30°C, harvested, washed and resuspended in sterile lake water. 500 ml filtered-autoclaved lake water microcosms were inoculated to give an initial viable count of approximately 10^7 cfu/ml. The flasks were incubated in the dark without shaking at 4°C, 15°C, 25°C, 30°C and 37°C for 13 days without any nutrient addition. Succinate dehydrogenase activity assay was carried out as described in Chapter 2. Activity is expressed as mM succinate oxidised/min/mg of cell protein.

Figure 5.11 NADH dehydrogenase activity of *Escherichia coli* in filtered-autoclaved lake water.

E. coli was grown in nutrient broth overnight at 30°C, harvested, washed and resuspended in sterile lake water. 500 ml filtered-sterile lake water microcosms were inoculated to give an initial viable count of approximately 10^7 cfu/ml. The flasks were incubated in the dark without shaking at 4°C, 15°C, 25°C, and 37°C for 13 days without any nutrient addition. NADH dehydrogenase activity assay was carried out as described in chapter 2. Activity was expressed as mM NADH oxidised min mg cell protein.

Figure 5.12 Amount of total protein in *Escherichia coli* in filtered-autoclaved lake water under starvation stress at different temperatures.

Total protein in *E. coli* cells was assayed as described in chapter 2.

Fig.5.10

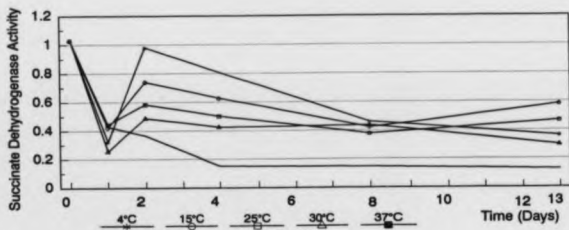


Fig.5.11

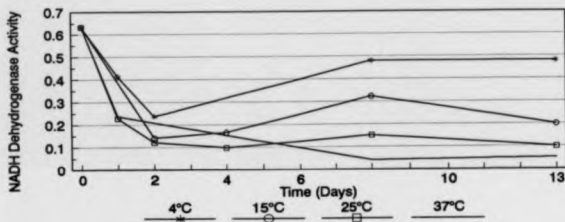


Fig.5.12

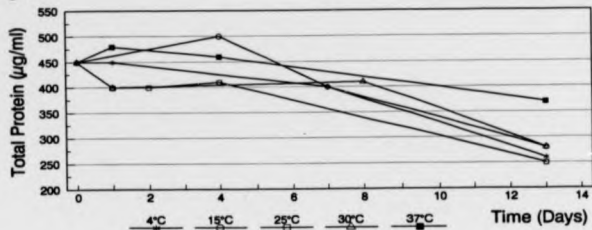


Figure 5.13 The effect of CCCP on succinate dehydrogenase activity of *Escherichia coli* in filtered-autoclaved lake water.

E. coli was grown in nutrient broth overnight at 30°C, harvested, washed and resuspended in sterile lake water. 500 ml filtered-autoclaved lake water microcosms were amended with CCCP at a final concentration of 5 μ M and 10 μ M. The flasks were inoculated to an initial viable count of approximately 10^8 cfu/ml. The flasks were incubated in the dark without shaking at 30°C for 7 days without any nutrient addition. Succinate dehydrogenase activity assay was carried out as described in chapter 2. Activity was expressed as mM succinate oxidised min mg of cell protein.

Figure 5.14 The effect of CCCP on NADH dehydrogenase activity of *Escherichia coli* in filtered-autoclaved lake water.

E. coli was grown in nutrient broth overnight at 30°C, harvested, washed and resuspended in sterile lake water. 500 ml filtered-autoclaved lake water microcosms were amended with CCCP at a final concentrations of 5 μ M and 10 μ M. The flasks were inoculated to give an initial viable count of approximately 10^8 cfu/ml. The flasks were incubated in the dark without shaking at 30°C for 7 days without any nutrient addition. NADH dehydrogenase activity assay was carried out as described in Chapter 2. Activity was expressed as mM NADH oxidised min mg of cell protein.

Fig.5.13

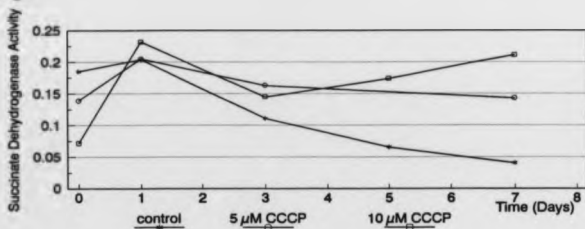
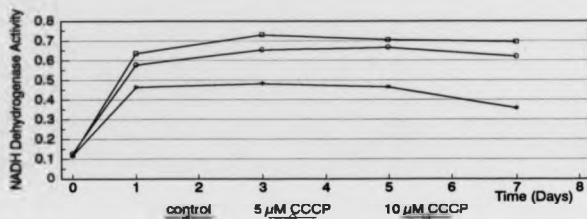


Fig.5.14

Total cellular Protein ($\mu\text{g/ml}$)

Days	control	5 μM CCCP	10 μM CCCP
0	1.015	1.062	0.959
1	1.013	1.006	1.028
3	1.032	1.0	1.031
5	1.052	1.032	1.034
7	1.057	1.035	1.033

Figure 5.15 The effect of sodium azide on the succinate dehydrogenase activity of *Escherichia coli* in filtered-autoclaved lake water.

E. coli was grown in nutrient broth overnight at 30°C, harvested, washed and resuspended in sterile lake water. 500 ml filtered-autoclaved lake water microcosms were amended at a final concentrations of 50 μ M and 100 μ M. The flask were inoculated to give an initial viable count of approximately 10^8 cfu/ml. The flasks were incubated in the dark without shaking at 30°C for 7 days without any nutrient addition. Succinate dehydrogenase activity assay was carried out as described in Chapter 2. Activity was expressed as mM succinate oxidised min mg of cell protein.

Figure 5.16 The effect of sodium azide on the NADH dehydrogenase activity of *Escherichia coli* in filtered-autoclaved lake water.

E. coli was grown in nutrient broth overnight at 30°C, harvested, washed and resuspended in sterile lake water. 500 ml filtered-autoclaved lake water microcosms were amended with sodium azide at a final concentration of 50 μ M and 100 μ M. The flasks were inoculated to give an initial viable count of approximately 10^8 cfu/ml. The flasks were incubated in the dark without shaking at 30°C for 7 days without any nutrient addition. NADH dehydrogenase activity assay was carried out as described in Chapter 2. Activity was expressed as mM NADH oxidised min mg cell protein.

Fig. 5.15

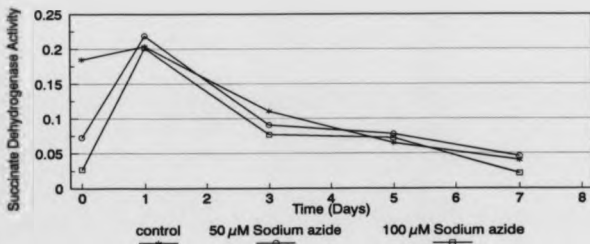
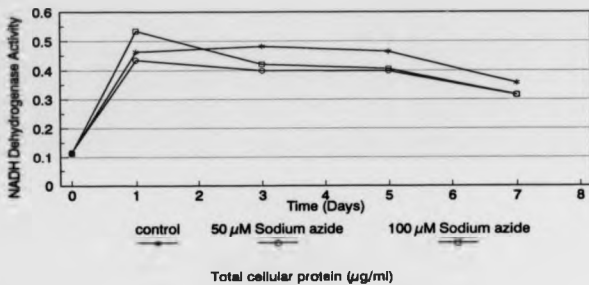


Fig. 5.16

Total cellular protein ($\mu\text{g/ml}$)

Days	control	50 μM azide	100 μM azide
0	1.015	.994	1.057
1	1.013	.967	1.038
3	1.032	.941	1.02
5	1.052	1.014	1.029
7	1.057	1.0	1.028

5.3 Discussion

The relation between morphological and physiological changes in bacterial cells and the viable but non-culturable state under nutrient limited conditions is an important question in microbial ecology. However an increased concentration of some nutrient sources can also result in cell death as result of which survival of bacteria is affected. This can be related to energy balance and the respiration rate of bacteria. Respiration rate has been measured in the cells as metabolic changes occur using variety of methods. The respiration rate can be a reflection of the metabolic activity of bacterial cells under a number of circumstances. One of the ways of examining metabolic activity is to use ETS activity as a measurement. This can be determined by means of the reduction of triphenyltetrazolium chloride. In this method the colourless oxidised INT chloride form is reduced to coloured formazan and deposited in the cells. This deposit can be extracted and quantified (Stolp, 1988). Initial studies showed that here ETS activity was increased when the initial inoculum size was increased in filtered-autoclaved lake water at 30°C. These results also showed that the deposition of formazan reached a maximum in the cells after 80 minutes of incubation. These experiments also showed that ETS activity reflects the total number of bacteria in the samples. Trevors (1982) suggested that intracellular INT reduction and formazan deposition occurred only in respiring cells and it was a function of respiratory activity. Therefore measuring the ETS activity monitors the rate of respiration in the bacterial cells in aquatic systems if incubated *in situ*. Similar results have previously shown by Tabor and Neihof (1982) that metabolic activity in aquatic bacteria can be linked to ETS activity. They have also reported that the estimation of respiration rate of a microbial population from the determination of ETS activity must also consider the physiological state of the population. Harrison (1976) suggested that the

respiration rate must be related to cell biomass in an environment exposed to rapid and radical changes.

Numerous studies have been conducted on respiration rate by different methods in bacteria under starvation stress but not by means of ETS activity. Boylen and Ensign (1970) reported that respiration rate of *Arthrobacter crystallopoideus* dropped sharply after 2 days of starvation then became undetectable. It has also been shown by Novitaky and Morita (1978) and Dawson *et al.* (1981) that marine vibrios reduced their rate of respiration very significantly under starvation stress. It is known that starved bacteria can synthesise new proteins and have the ability to respond to changes in the environment (Amy and Morita, 1983; Kjelleberg *et al.*, 1982). They also suggested that although dwarf bacteria remained viable but non-culturable in marine environments, they contained high concentrations of ATP and other nucleoside triphosphates (Morita, 1982; Smigielsky *et al.*, 1989). Here it was found, although ETS activity in *E.coli* was higher at 30°C than at 4°C or 15°C in filtered-autoclaved lake water, activity declined by about 65% in a 24 h starvation period. However this decrease in ETS activity in *E.coli* only started after 6 days of incubation at 4°C and 15°C. Trevors (1984) has also shown that the minimum loss of ETS activity took place at 4°C and maximum at 37°C with these results are correlated with the other findings here that viability of *E.coli* declined faster at 37°C than at the other temperatures and remained highest at 4°C in filtered-autoclaved lake water under starvation stress. Also a significant cell size reduction occurred at 37°C in filtered-autoclaved lake water and at not 4°C. These alterations in *E.coli* cells are probably a chain-like relationship responding to changes in temperature and nutrient conditions giving bacteria an optimum strategy to protect themselves against starvation stress. One more important point can be deduced from these results that although viability of *E.coli* remained unchanged in lake water at 4°C and 15°C, ETS activity began to decline after 6 days of incubation. On the other hand, at 30°C viability

decline below $10^{4.5}$ cfu/ml but there was still measurable respiration activity in *E. coli* cells. This means that some of the cells remained viable but non-culturable but still capable of respiration in lake water. Harrison (1976) stated that an increase in growth rate requires synthesis of a great amount of RNA and some other regulatory molecules whose concentrations are limiting to growth and respiration at the lower temperatures. It is known that optimum temperature for growth of this *E. coli* is around at 30°C. This means that at this temperature *E. coli* probably consumes a large amount of energy for respiration rate and consequently ETS activity will slow down after a short time to maintain cell energy if faced with starvation stress. Here *E. coli* was faced with these circumstances in lake water at 30°C, as a result ETS activity declined sharply within a day of starvation.

The respiration rate in the bacterial cells is an important function for energy production which is required to drive growth processes under different nutrient conditions. Atkinson (1968) suggested that an increase in respiration rate must effect a severe imbalance in the cell's energy charge. The energy status of living cells is recorded in terms of their adenylate energy charge (Atkinson, 1968). This is expressed as $([ATP] + 0.5 [ADP]) / ([ATP] + [ADP] + [AMP])$ which for normal growth and metabolism must be maintained within the range of 0.8 to 0.95 (Atkinson, 1977). There is a relationship between viability, adenylate energy charge and cell size reduction. Chapman *et al.* (1971) showed that *E. coli* grew only when its energy charge exceeded 0.8, that viability was maintained between 0.5 and 0.8 and that death occurred below values of 0.5. ETS activity probably reflects the metabolic energy changes in the cell. Therefore the addition of single carbon sources may affect metabolism of the *E. coli* cells and some of them probably cause severe imbalance. As a result they accelerated death of starved bacteria where prolonged survival is associated with a low rate of endogenous metabolism more closely matched to the maintenance energy requirement (Gray, 1976). It has been investigated in this

study that some nutrient sources, especially carbon sources, enabled *E. coli* to grow in lake water but then accelerated the rate of disappearance of the cells. There might be a link between respiration rate, utilisation of nutrient sources and the survival of *E. coli*. Our results demonstrated that some carbon sources, such as sucrose, fructose and succinate, resulted in an increase in ETS activity of *E. coli* in filtered-autoclaved lake water at 15°C and at 30°C but at 37°C the rate of increase in respiration rate was higher. Here it has been shown in a long-term experiments sucrose, fructose and succinate led to a decline the survival of *E. coli* in lake water at 30°C. On the other hand nitrogen sources such as ammonium sulphate, ammonium nitrate and casein resulted in prolonged survival of *E. coli* but ETS activity was not higher than in the controls. In addition, ETS activity was increased by the addition of increased concentration of lactose, leucine and proline to the filtered-autoclaved lake water.

Another part of our experiments also demonstrated that ETS activity after 2, 4, 7 and 16 days of starvation was increased in an order $2 < 4 < 7 < 16$ days after addition of nutrient broth to the filtered-autoclaved lake water at 15°C and 30°C. Positive relationships were identified between the length of starvation and ETS activity. The ETS activity in *E. coli* occurred the lowest after 2 days of starvation and the highest after 16 days of starvation after the addition of nutrient broth to the filtered-autoclaved lake water at 15°C and 37°C. The ETS activity measurements were carried out three times (2, 6, 24 h) after nutrient broth addition every sampling day (2, 4, 7 and 16 days). Usually the highest activity was detected at 6 h after the amendments. Although ETS activity was increased in every phase of growth, this did not occur after 24 h incubation at 30°C. These results suggest that the nutrient scavenging capacity of *E. coli* was improved by starvation stress and nutrient utilisation from lake water could be faster in response to long-term nutrient deprivation conditions. Second important outcome from these results is that again *E. coli* showed a reduced respiration rate or ETS activity at 30°C compared to cells incubated at 15°C and 4°C.

Another way of detecting respiration rate of bacteria is to measure activity of enzymes involved such as succinate and NADH dehydrogenase (Smigielsky *et al.*, 1989, 1990). Smigielsky *et al.* (1989) showed that respiration rate declined after 2-3 days of starvation at the same time respiratory potential of *Vibrio sp.* decreased 4-5 folds as a result of a decrease in succinate dehydrogenase and NADH dehydrogenase activity. Our experiments here demonstrated that the activity of succinate and NADH dehydrogenase was temperature dependent as was the viability of *E. coli*, cell size reduction of *E. coli* and ETS activity. In chapter 3 it was shown that loss of colony forming ability occurred at the fastest rate at 37°C and lowest at 4°C. It is possible that *E. coli* enters a viable but non-culturable state at 37°C. Cell size reduction was also highest at 37°C and the lowest at 4°C. Moreover succinate dehydrogenase and NADH dehydrogenase activity declined to the lowest activity level after 13 days of starvation period at 37°C compare to 4°C, 15°C, 25°C and 30°C incubated samples in filtered-autoclaved lake water. On the contrary the highest activity of respiratory enzymes in *E. coli* cells occurred at 4°C after 13 days incubation. In general, activity of these two dehydrogenases took place from highest to lowest at temperature 4 > 15 > 25 > 30 > 37°C.

The addition of a sublethal concentration of CCCP, which functions as an uncoupling agent, to the lake water led to an increase in succinate and NADH dehydrogenase activity whereas the viability of *E. coli* declined in amended lake water. Smigielsky *et al.* (1990) suggested that the addition of CCCP to the culture of *Vibrio sp.* led to defects in the respiratory chain and prevented size reduction during periods of nutrient limitation. Our results showed that there was no cell size changes after the addition of 5 μ to 10 μ M CCCP to the filtered-autoclaved lake water. Decline in numbers of *E. coli* could be due to the changes in pH gradients and respiration without generating ATP for the active process. Besides, sodium azide did not cause any alteration in dehydrogenase

activity in *E.coli* but led to the decline in number of cells in filtered-autoclaved lake water at a final concentration higher than 50 μ M.

In brief, here we explored the changes in metabolic activity of *E.coli* by means of ETS activity and succinate and NADH dehydrogenase activity under starvation stress. At the same time ETS activity of *E.coli* was examined by the additions of single carbon and nitrogen sources to the filtered-autoclaved lake water. It was found here that there was a strong correlation between starvation survival of *E.coli*, ETS activity, succinate and NADH dehydrogenase activity under starvation stress especially at 30°C. Cell volume changes were also correlated to viability of *E.coli* and activity of the dehydrogenases enzymes. However when colony forming units declined below detection limit, ETS activity was still detectable in *E.coli* cells.

**VI. The effects of stress on alkaline phosphatase activity and survival of
*Escherichia coli***

6.1. Introduction

Phosphorus is an essential element for the growth and reproduction of bacteria. Phosphorus and its compounds play a very significant role in many aspects of cell metabolism. Inorganic phosphate is abundant in some environments but natural waters also contain many different organic phosphate containing compounds. These can be of biological origin or products of human activities. Phosphorus-containing organic compounds account for more than 50% of the total amount of this element in aquatic environments (Corner and Davies, 1971). Organic phosphates are not directly utilised by microorganisms. They have to be hydrolysed by enzyme action outside the microbial cell before the phosphate moiety is utilizable (Wynne, 1977).

A major enzyme involved in the degradation of organic phosphatases is alkaline phosphatase. This enzyme is synthesised usually under conditions where inorganic phosphate is limiting but where the cell is still able to grow, i.e. there is sufficient carbon, nitrogen and other essential nutrients available. Inorganic phosphate limitation results in the synthesis of a battery of proteins, including alkaline phosphatase, important for scavenging phosphate from the environment (Filloux *et al.*, 1988).

In *E.coli* alkaline phosphatase is located in the periplasmic space between the outer membrane and the cytoplasmic membrane (Heppel, 1967). The enzyme may be liberated from *E.coli* cells either by conversion of the cells to spheroplasts (Malamy and Horecker, 1961) or by osmotic shock (Heppel, 1967). In a species of *Pseudomonas* the removal of a loosely bound layer of the cell wall by washing with sodium chloride leads to the release of large amounts of alkaline phosphatase activity. Phosphatases have been shown to be associated with the cell membrane by ultrastructural cytochemistry (Tinglu *et al.*, 1984).

The activity of alkaline phosphatase and other phosphatases has been studied in natural environments where the enzyme plays a key role in the

mineralization of phosphate compounds and hence in maintaining the ecological equilibrium (Jorgensen, 1976; Suida, 1984). However the effects of changing environmental conditions on alkaline phosphatase activity have been considered only briefly (Matavulj and Flint, 1987). Little is known about alkaline phosphatase activity in cells experiencing long-term starvation or when the cell enters the viable but non-culturable state. In cells grown in the laboratory under phosphate limitation, alkaline phosphatase can account for up to 6 % cellular protein (Garen and Levinthal, 1960) However, it is still not accepted that alkaline phosphatase activity can be derepressed under environmental conditions although Matavulj and Flint (1987) showed that there was a higher specific activity associated with cells from low phosphate environments compared to those from high phosphate environments.

There have been studies linking alkaline phosphatase activity and the survival of bacteria in phosphate depleted environments. Gauthier *et al.* (1990) have shown that some enzyme activities, including alkaline phosphatase activity, increased in *E. coli* cells in nutrient-free seawater. This they ascribed to nutrient starvation. They have suggested that the long-term survival of *E. coli* in seawater is dependent upon the ability of the bacteria to produce alkaline phosphatase although do not suggest a function for this enzyme under starvation conditions. They also reported that glycinebetaine which is an osmoprotectant enhanced alkaline phosphatase activity *E. coli* in seawater. A similar result was shown by Munro *et al.* (1989).

Phosphate starvation is not the only factor which can lead to derepression of alkaline phosphatase in bacteria. Physiological conditions which lead to changes in internal nucleotide pools can also lead to derepression even in the presence of high phosphate concentration inside the cell or in the external medium (Wilkins, 1972). Alkaline phosphatase was derepressed in a thymine auxotroph of *E. coli* grown in the absence of thymine or uracil even in the presence of high phosphate concentrations (Wilkins). Alkaline phosphatase

activity was also increased by the addition of glucose, or glucose and ammonium nitrate to soil samples (Gadkari, 1984). This may have been the results of derepression but it is possible that the increase in activity was simply a reflection of the increased biomass in soils amended with easily metabolised substrates such as carbohydrates. Gadkari (1984) also showed that the addition of low concentrations of pesticides, such as Goltrix, to soil also resulted in increased alkaline phosphatase activity. Again this could be the result of an increase in microbial biomass. The addition of high concentrations of goltrix led to a decrease in alkaline phosphatase activity. Phosphate starvation does not only derepress alkaline phosphatase synthesis; it also leads to the synthesis of a number of other proteins some of which are involved in phosphate transport through the outer and cytoplasmic membranes of the Gram-negative cell. Wackett *et al.* (1987) showed that when *E.coli* became phosphate limited, the synthesis of 81 proteins was markedly changed. About 50% of these proteins were detected only under conditions of phosphate limitation. The composition of the outer membrane proteins, especially porins, of *E.coli* also changed in response to the changes in environmental conditions leading to an increase in permeability of the cells (Forst and Inouye, 1988; Csonka, 1989). A number of environmental parameters have been shown to affect the regulation of porins such as medium osmolarity (Kawaji *et al.*, 1979), temperature (Lugtenberg *et al.*, 1976), and carbon sources (Scott and Harwood, 1980). Changes in the outer membrane porin, PhoE, have been correlated with the synthesis of alkaline phosphatase under conditions of phosphate starvation (Kawaji *et al.*, 1979). PhoE is an anion-specific porin which mediates the passage of phosphate across the outer membrane into the periplasm (Nikaido and Vaara, 1985). With the aid of alkaline phosphatase, PhoE delivers usable phosphate to the transport systems in the cytoplasmic membrane (Amemura *et al.*, 1985; Nakata *et al.*, 1987; Elvin *et al.*, 1987). This chapter examines the role of alkaline phosphatase activity in the survival of *E.coli* in filtered-autoclaved lake water under conditions of

starvation stress, osmotic stress, heat shock and oxidation stress. The changes in alkaline phosphates activity seen over a period of time at different temperatures were monitored. The aim was to determine whether alkaline phosphatase activity was influenced by environmental factors other than phosphate concentration and to establish a link between survival and alkaline phosphatase activity in filtered-autoclaved lake water microcosms.

6.2. Results

6.2.1. Alkaline Phosphatase Activity of *Escherichia coli* starved in filtered-autoclaved lake water at different temperatures.

In this experiment the alkaline phosphatase activity of *E.coli* grown in a low phosphate mineral medium was examined in cells resuspended in filtered autoclaved lake water held at different temperatures. Previous results have suggested that this lake water does not contain enough nutrients to allow *E.coli* to grow at any of the temperatures used. Hence it was assumed that the cells were under a starvation stress. Alkaline phosphatase in this strain of *E.coli* is known to be derepressible and produced only under conditions of phosphate limitation i.e. a form of starvation stress.

Alkaline phosphatase activity was measured using a spectrophotometric assay over a period of 70 d starvation at temperatures between 4°C and 37°C. The results were expressed as total alkaline phosphatase activity of the sample or as specific alkaline phosphatase activity per unit of viable count. Fig 6.1 shows that alkaline phosphatase activity was relatively stable over the course of starvation period. There was an increase in total activity over the starvation period initially at 4°C and 15°C but after 26 days at all the temperature studied. Total alkaline phosphatase activity increased by 160.5% at 4°C 137.1% at 15°C, 163.5% at 25°C 128.2% at 30°C and 155.7% at 37°C over the 70 d starvation period compared to the 0 d control. Although the activity increased the viable count remained constant at all temperatures except 37°C where the viable count fell below detection limits within 10 days. Despite this loss in viable count the alkaline phosphatase activity in the microcosm held at 37°C increased after the viable count was effectively zero. With the viable count declining expression of the results as specific activity would have shown an even more dramatic rise in alkaline phosphatase activity at 37°C. Because total alkaline phosphatase activity increased with no increase in viable count the

results suggest that starvation could lead to derepression of alkaline phosphatase in *E. coli* in filtered-autoclaved lake water.

The experiment was repeated using distilled water as a more severe starvation stress than filtered-autoclaved lake water. Again there was an increase in alkaline phosphatase activity especially at 30°C and 37°C over a 20 days period (Fig 6.2). Again this increase in alkaline phosphatase activity was accompanied by a decline in the viable count as the cells became more stressed. At the lower temperatures the increase was not as pronounced as at 30°C and 37°C.

6.2.2 The effects of nutrient sources on alkaline phosphatase activity of *Escherichia coli* in filtered-autoclaved lake water.

6.2.2.1 The effect of amendment amino acids on alkaline phosphatase activity

E. coli grown in low phosphate minimal medium was inoculated into filtered-autoclaved lake water amended with a variety of amino acids which had some effect on the survival of *E. coli* (Chapter 4). The alkaline phosphatase activity of the cells was monitored over a period of at least 20 d during incubation of the microcosms at 15°C. The results suggested that some amino acids significantly altered the alkaline phosphatase activity of *E. coli* in filtered-autoclaved lake water. Arginine, methionine, serine, isoleucine and phenylalanine did not cause any significant changes in the alkaline phosphatase activity in amended flasks compared to the unamended control (Fig 6.3). All showed the same increase in activity over the first 24 h probably due to the small increase in viable count seen during this time period. The activity then remained constant for the 14 d incubation period. However, flasks amended

Figure 6.1 Alkaline phosphatase activity of *Escherichia coli* under starvation stress in filtered-autoclaved lake water at different temperatures.

E. coli was grown in a low phosphate minimal medium overnight at 30°C. The cells were harvested, washed and resuspended in sterile lake water. 100 ml distilled water microcosms were inoculated to give an initial viable count of 10^6 cfu/ml. The flasks were incubated in the dark without shaking at 4°C, 15°C, 25°C, 30°C and 37°C. Alkaline phosphatase activity was measured by removing aliquots, buffering the pH 9.0 with Tris-buffer, and using pNPP as a colorimetric substrate. Activity assays were performed at 30°C. Viable counts were determined on a surface spread plates after overnight incubation at 37°C.

Activity is expressed as change in absorbance at 420 nm/3 h. These can be converted to μM pNP released/ml/hr by multiplying by 15.7.

Figure 6.2 Alkaline phosphatase activity of *Escherichia coli* in distilled water under starvation at different temperatures

E. coli was grown in a low phosphate minimal medium overnight at 30°C. The cells were harvested, washed and resuspended in sterile lake water. 100 ml distilled water microcosms were inoculated to give an initial viable count of 10^6 cfu/ml. The flasks were incubated in the dark without shaking at 4°C, 15°C, 25°C, 30°C and 37°C. Alkaline phosphatase activity was measured by removing aliquots, buffering the pH 9.0 with Tris-buffer, and using pNPP as a colorimetric substrate. Activity assays were performed at 30°C. Viable counts were determined on a surface spread plates after overnight incubation at 37°C. Total alkaline phosphatase activity is expressed as in Fig. 6.1.

Total Alkaline Phosphatase Activity

Figure 6.1

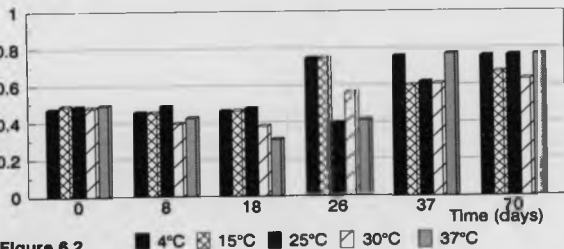
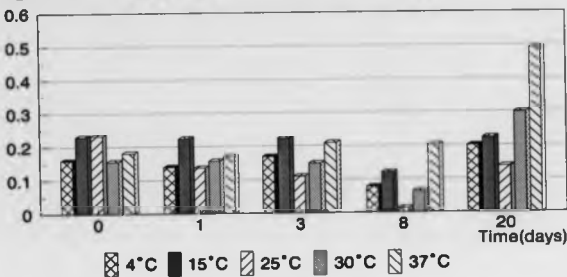


Figure 6.2



For fig. 6.1		Plate counts (Log. cfu/ml)		For fig. 6.2	
days	37°C	days	37°C		
0	5.82	0	6.36		
8	5.92	1	5.84		
18	5.3	3	5.51		
26	5.0	8	5		
37	4.2	20	<2		
70	<1				

with histidine showed a decrease in activity over the incubation period although there was no change in the viable count (Fig 6.4). Flasks amended with cysteine showed a total alkaline phosphatase activity below the control although the total activity in the microcosm remained virtually constant (Fig 6.4). Flasks amended with glycine showed an initial increase along with the control but the final activity had begun to decline after 14 d incubation (Fig 6.4). Again the viable count had remained at a constant value after the first 24 h incubation period.

Specific activity calculations show that increasing concentrations of glutamate, and glycine led to further derepression of alkaline phosphatase activity but increasing concentration of proline led to a reduction in alkaline phosphatase activity (Table 6.1). Histidine and cysteine were apparently inhibitory to alkaline phosphatase activity.

In the experiment above the increased derepression of alkaline phosphatase in cells with an already derepressed enzyme by amendment of filtered-autoclaved lake water with amino acids was shown. Derepression of enzyme synthesis in *E.coli* grown in high phosphate minimal medium which represses alkaline phosphatase synthesis after inoculation into amino acid-amended lake water was also shown. Most amino acids had no effect on alkaline phosphatase activity (Fig 6.6) with total alkaline phosphatase activity remaining constant over the 23 d incubation period at 15°C. However both proline and glutamate caused an apparent increase in the specific activity of alkaline phosphatase over 18 d incubation period (Fig 6.5). Activity is expressed as specific activity in these samples because the viable count increased over the same period. Derepression is suggested because there was at least a doubling in the specific activity compared to the control. This could only occur if new enzyme was being synthesised in these samples.

6.2.2 Carbon Sources

E.coli grown in high phosphate minimal medium was inoculated into filtered-autoclaved lake water amended with a variety of carbon sources. Again

these organisms showed derepression of alkaline phosphatase activity over a period of starvation of up to 13 d. Fig 6.7 shows the effects of amendment of filtered-autoclaved lake water with maltose, glycerol, and lactate on specific activity of *E.coli*. Glucose had an immediate effect on specific activity which increased rapidly after inoculation. The other carbon sources showed smaller increases but all had specific alkaline phosphatase activity the unamended control by the end of the starvation period. Glucose caused at an increase of three times in specific activity. In a separate experiment amendment of filtered-autoclaved lake water with sucrose, fructose, glycerol, succinate and acetate was carried out (Fig 6.8). Again all carbon sources increased the specific alkaline phosphatase activity of the cells over the unamended control. Some of the carbon sources had also been shown to increase the survival times of *E.coli* in filtered-autoclaved lake water (Chapter 4).

The effects of increasing the concentration of the carbon source used to amend the filtered-autoclaved lake water are shown in Table 6.2. Generally increasing concentration of sucrose, glycerol, succinate, acetate and to a lesser degree fructose and lactose led to a decrease in the specific alkaline phosphatase activity although all remain higher than the control. Glycerol and acetate at 2 mg-C/l had the most startling effect increasing the specific activity to nearly ten times that of the amended control.

The viable count in these samples also increased showing that *E.coli* could grow under these conditions. Because no phosphate source was added to the filtered-autoclaved lake water it is perhaps not surprising that alkaline phosphatase activity was derepressed by the addition of carbon sources. To the cells this situation would be similar to being grown in a phosphate limited medium. In some instances, however, no increase in cell numbers was detected but derepression still occurred indicating that the cells were still capable of enzyme synthesis if not of cellular biomass or numbers.

Figure 6.3 Amino acids effect on alkaline phosphatase activity of low phosphate minimal medium grown *Escherichia coli* at 15°C.

Figure 6.4 L-cysteine, L-histidine, and glycine effect on alkaline phosphatase activity of *Escherichia coli* in filtered -autoclaved lake water at 15°C

E. coli was grown in a low phosphate minimal medium overnight at 30°C. The cells were harvested, washed and resuspended in sterile lake water. 100 ml filtered-autoclaved lake water microcosms were inoculated to give an initial viable count of 10^7 cfu/ml. The flasks were incubated in the dark without shaking at 15°C. Flasks were amended with amino acids at final concentrations of 2 mg/l. Alkaline phosphatase activity was measured by removing aliquots, buffering the pH 9.0 with Tris-buffer, and using pNPP as a colorimetric substrate. Activity assays were performed at 30°C. Viable counts were determined on a surface spread plates after overnight incubation at 37°C. Total alkaline phosphatase activity is expressed as change in absorbance at 420 nm/3 h. These can be converted to μM pNP released/ml/h by multiplying by 15.7.

Figure 6.3

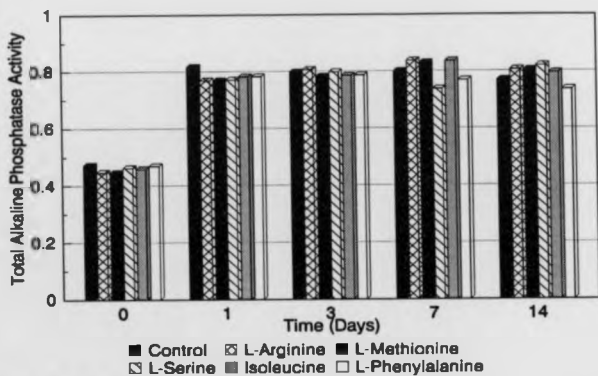


Figure 6.4

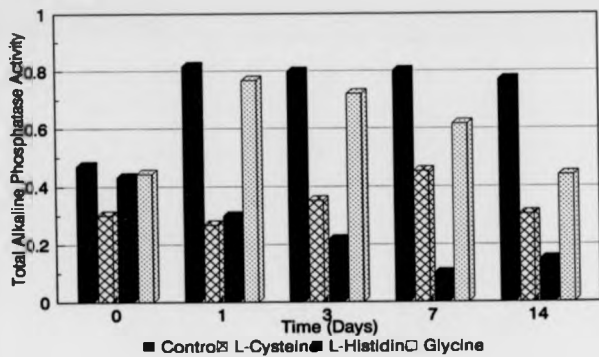


Figure 6.5 The effect of proline and glutamate on alkaline phosphatase activity in high phosphate-grown *Escherichia coli* cells in filtered-autoclaved lake water.

Figure 6.6 The effect of L-lysine, Tryptophan, asparagine, L-tyrosine and L-serine on alkaline phosphatase activity in high phosphate medium grown *Escherichia coli* in filtered-autoclaved lake water.

E. coli was grown in a low phosphate minimal medium overnight at 30°C. The cells were harvested, washed and resuspended in sterile lake water. 100 ml filtered-autoclaved lake water microcosms were inoculated to give an initial viable count of 10^7 cfu/ml. The flasks were incubated in the dark without shaking at 15°C. Flasks were amended with amino acids at final concentrations of 2 mg/l. Alkaline phosphatase activity was measured by removing aliquots, buffering the pH 9.0 with Tris-buffer, and using pNPP as a colorimetric substrate. Activity assays were performed at 30°C. Viable counts were determined on a surface spread plates after overnight incubation at 37°C. Total alkaline phosphatase activity is expressed as change in absorbance at 420 nm/3 h. These can be converted to μM pNP released/ml/h by multiplying by 15.7. Specific alkaline phosphatase activity was expressed as unit of enzyme activity for per cell by means of dividing optical density to viable count.

Figure 6.5

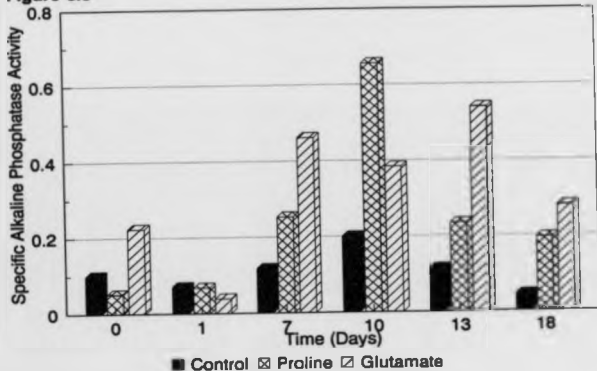


Figure 6.6

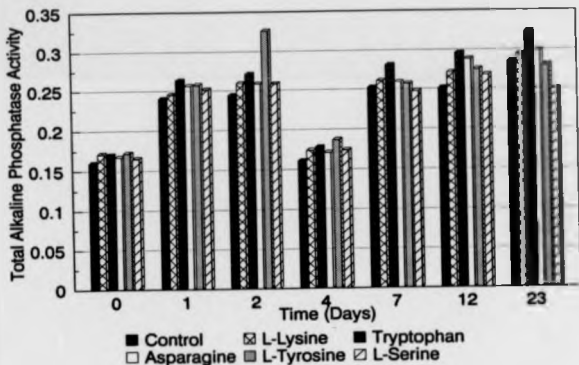


Table 6.1 The effects of different amino acids concentrations on alkaline phosphatase activity of low phosphate medium grown *Escherichia coli* in filtered-autoclaved lake water at 15°C.

Amino-acid Concentrations	SAPA	Changes in SAPA as Percentages
Control	1.350	
Glutamate (2.5 mg/L)	1.720	127.40
Glutamate (5 mg/L)	2.370	172.90
Glutamate (7.5 mg/L)	2.840	210.30
Glycine (2.5 mg/L)	4.610	341.40
Glycine (5 mg/L)	5.570	412.50
Glycine (7.5 mg/L)	6.620	490.30
L-Histidine(2.5 mg/L)	0.239	17.70
L-Histidine(5 mg/L)	0.310	22.90
L-Histidine(7.5 mg/L)	0.190	14.07
Cysteine (2.5 mg/L)	0.400	29.60
Cysteine (7.5 mg/L)	0.590	43.70
Proline (2.5 mg/L)	1.510	111.8
Proline (5 mg/L)	1.240	91.8
Proline (7.5 mg/L)	0.040	2.96

6.2.3 The effects of osmotic stress and osmoprotectants on the alkaline phosphatase activity of *Escherichia coli* under starvation stress.

The results describe above suggest that alkaline phosphatase may be synthesised as a response to starvation stress. In this section the synthesis of alkaline phosphatase in response to other stresses is examined. The role of osmotic shock in the derepression of alkaline phosphatase activity in *E. coli* was shown by transferring *E. coli* grown in phosphate-limited minimal medium to filtered-autoclaved lake water supplemented with different concentrations of sodium chloride. Fig 6.9 shows that increasing the concentration of sodium chloride in filtered-autoclaved lake water from 0.17 mM to 34.2 mM increased alkaline phosphatase activity. The alkaline phosphatase activity was increased to 109.9% of the control with 0.17 mM additions, 125.3% with 1.71 mM, 138.8% with 8.65 mM, 226.3% with 17.1 mM and 246.8% with 34.2 mM additions after 10 days incubation in filtered-autoclaved lake water at 30°C. The increase in activity was not matched by an increase in viable count. Indeed in the highest concentrations of sodium chloride the viable count had declined to undetectable levels within 1 d. Hence if results had been expressed as specific activity the increase in activity would have been seen to have been higher.

The results suggest that alkaline phosphatases might be derepressed by changing osmolarity of the filtered-autoclaved lake water. This increase could be due to true derepression of enzyme synthesis or to changes in the outer membrane of *Escherichia coli* leading to easier access for enzyme substrate to the periplasmic space where alkaline phosphatase is located.

The ability of bacteria to adapt to fluctuations in the ambient osmolarity of an aquatic environment is of fundamental importance for their survival (Munro *et al.*, 1989). In *E. coli* this is achieved through osmoregulatory processes that produce a cytoplasm which has both an optimal osmotic pressure

Figure 6.7 The effect of different carbon sources (maltose, glucose, glycerol, lactose) on the specific alkaline phosphatase activity of high phosphate-grown *Escherichia coli* in filtered-autoclaved lake water at 15°C.

Figure 6.8 The effect of different carbon sources (sucrose, fructose, glycerol, succinate, acetate) on the specific alkaline phosphatase activity of high phosphate-grown *Escherichia coli* in filtered-autoclaved lake water at 15°C.

E. coli was grown in a low phosphate minimal medium overnight at 30°C. The cells were harvested, washed and resuspended in sterile lake water. 100 ml filtered-autoclaved lake water microcosms were inoculated to give an initial viable count of 10^7 cfu/ml. The flasks were incubated in the dark without shaking at 15°C. Flasks were amended with carbon sources at final concentrations of 2 mg-C/l. Alkaline phosphatase activity was measured by removing aliquots, buffering the pH 9.0 with Tris-buffer, and using pNPP as a colorimetric substrate. Activity assays were performed at 30°C. Viable counts were determined on a surface spread plates after overnight incubation at 37°C. Specific alkaline phosphatase activity was expressed as unit of enzyme activity for per cell by means of dividing optical density to viable count. These can be converted to μM pNP released/ml/h by multiplying by 15.7.

Figure 6.7

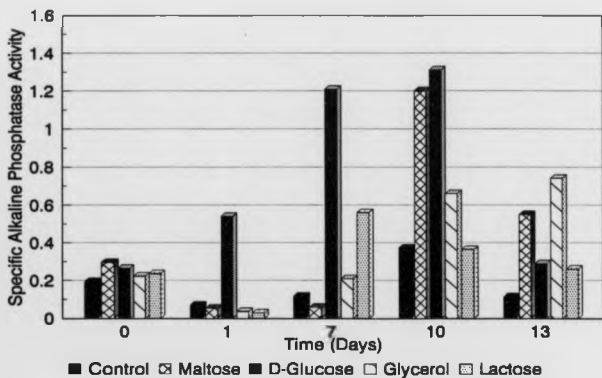


Figure 6.8

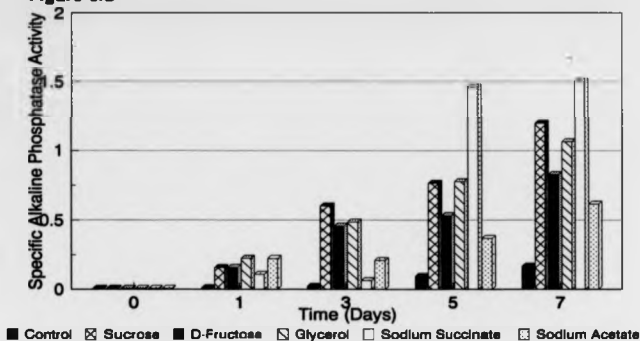


Table 6.2 Alkaline Phosphatase Activity of High Phosphate-Grown *Escherichia coli* in filtered-autoclaved lake water amended with different carbon sources

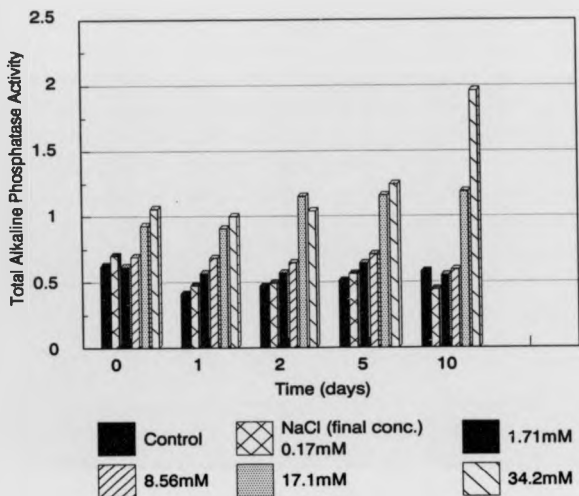
Carbon Source Concentrations	specific activity	Specific activity changes in (%)
Control	0.169	
Sucrose (2 mg-C/L)	1.560	923.0
(4 mg-C/L)	1.240	733.0
(6 mg-C/L)	1.062	628.4
Fructose (2 mg-C/L)	0.882	521.8
(4 mg-C/L)	1.482	876.9
(6 mg-C/L)	1.066	630.7
Glycerol(2 mg-C/L)	18.600	11,005.0
(4 mg-C/L)	11.370	6,727.0
(6 mg-C/L)	8.910	5,272.0
Succinate(0.88 mg-C/L)	14.540	8,603.0
(1.76 mg-C/L)	5.606	3,317.0
(2.64 mg-C/L)	2.052	1,214.0
Acetate (2 mg-C/L)	15.860	9,384.0
(4 mg-C/L)	0.413	244.3
(6 mg-C/L)	0.184	108.8
Lactose (2 mg-C/L)	1.195	707.1
(4 mg-C/L)	2.750	1,627.2
(6 mg-C/L)	0.870	514.7

Alkaline phosphatase activity was measured after 11 days of incubation.

Figure 6.9 The effect of osmotic stress on alkaline phosphatase activity of *Escherichia coli* in filtered-autoclaved lake water at 30°C.

E. coli was grown in a low phosphate minimal medium overnight at 30°C. The cells were harvested, washed and resuspended in sterile lake water. 100 ml filtered-autoclaved lake water microcosms were inoculated to give an initial viable count of 10^6 cfu/ml. The filtered-autoclaved lake water samples were amended with sodium chloride at concentrations from 0.17 mM to 34.2 mM final concentrations. The flasks were incubated in the dark without shaking at 30°C. Alkaline phosphatase activity was measured by removing aliquots, buffering the pH 9.0 with Tris-buffer, and using pNPP as a colorimetric substrate. Activity assays were performed at 30°C. Viable counts were determined on a surface spread plates after overnight incubation at 37°C. Total alkaline phosphatase activity is expressed as change in absorbance at 420 nm/3 h. These can be converted to μM pNP released/ml/h by multiplying by 15.7.

Figure 6.9



Viable count log(cfu/ml)

	control	0.17 mM	1.71 mM	8.65 mM	17.1 mM	34.2 mM
days						
0	6.0	5.84	5.84	5.65	5.56	5.98
1	5.96	5.64	5.8	5.6	< 1	<2.2
2	5.86	5.6	5.85	5.61	none	none
5	5.85	5.75	5.8	5.6	none	none
10	5.32	5.11	5.32	5	none	none

and is conducive to enzyme function (Booth *et al.*, 1988). Some osmoprotectants such as glycinebetaine are able to protect cells from decay in seawater (Munro *et al.*, 1989). Here the effects of osmoprotectants was examined on the alkaline phosphatase activity of derepressed *E.coli* in filtered-autoclaved lake water amended with 34.4 mM sodium chloride. The osmoprotectants used included glutamate, glycinebetaine, proline, acetyl- β -methylcholine and phosphorylcholine all caused a reduction in alkaline phosphatase activity of about 25% compared to the control. The two amino acids caused only a slight decrease in activity. Viable counts revealed that the rate at which *E.coli* declined in the amended filtered-autoclaved lake water was reduced in the presence of glycinebetaine, acetyl- β -methylcholine and phosphorylcholine. The results suggest that alleviation of some of the effects of osmotic stress by the use of osmoprotectants reduced the derepression of alkaline phosphatase in *E.coli*.

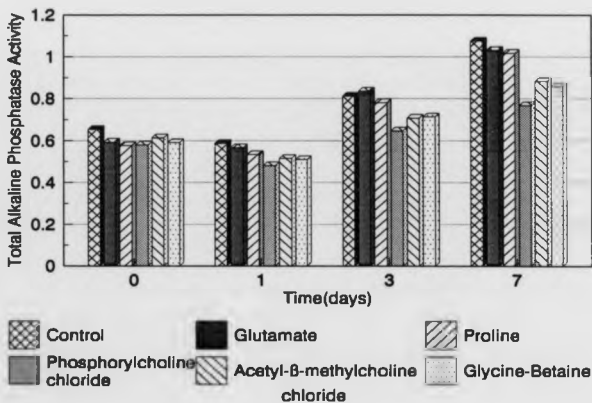
6.2.4 The effect of pH stress on alkaline phosphatase activity of *Escherichia coli* in filtered-autoclaved lake water.

The pH optimum for alkaline phosphatase activity in lake water is between 8.7 and 9.0 (Reichardt *et al.*, 1967). The survival of *E.coli* in filtered-autoclaved lake water may be influenced by the pH of the water and the alkaline phosphatase activity of the cells may also be affected by changes in pH. The pH of lake water changes with the season because of algal activity (Matavulj and Flint, 1987). The survival of *E.coli* in lake water therefore may be affected by seasonal changes to the pH of the water. Here *E.coli* was resuspended in filtered-autoclaved lake water which had its pH altered in the range 5.5 to 9.0. At pH 7.0 and 9.0 the final alkaline phosphatase activity was higher than samples kept at acidic pH (Fig 6.11). In general alkaline phosphatase activity was always higher in samples kept at pH 9.0 rather than any other pH. There was no indication that pH stress led to any derepression of alkaline phosphatase synthesis. The increase in alkaline phosphatase activity in the samples kept at

Figure 10 The effects of osmoprotectants on alkaline phosphatase activity of *Escherichia coli* in filtered-autoclaved lake water amended with sodium chloride.

E. coli was grown in a low phosphate minimal medium overnight at 30°C. The cells were harvested, washed and resuspended in sterile lake water. 100 ml filtered-autoclaved lake water microcosms were inoculated to give an initial viable count of 10^7 cfu/ml. The filtered-autoclaved lake water microcosms were amended with sodium chloride at concentrations of 34.2 mM and with the following osmoprotectants; glutamate (2.9 mM), proline (4.4 mM), phosphorylcholine chloride (0.77 mM), acetyl- β -methylcholine chloride (1 mM) and glycinebetaine (1.7 mM). The flasks were incubated in the dark without shaking at 15°C. Alkaline phosphatase activity was measured by removing aliquots, buffering the pH 9.0 with Tris-buffer, and using pNPP as a colorimetric substrate. Activity assays were performed at 30°C. Viable counts were determined on a surface spread plates after overnight incubation at 37°C. Total alkaline phosphatase activity is expressed as change in absorbance at 420 nm/3 h. These can be converted to μ M pNP released/ml/h by multiplying by 15.7.

Figure 6.10



pH 5.5 to 6.5 was similar to the changes in alkaline phosphatase activity seen previously; the subsequent loss of activity could simply have been a reflection of the instability of alkaline phosphatase at acid pH (Flint, 1974). pH changes seem to have little effect on alkaline phosphatase activity in *E.coli* under starvation conditions but the effects of pH on alkaline phosphatase activity are more complex than investigated here with the composition of the buffer and medium playing a role in the stability of the enzyme. Although enzyme synthesis may have been further derepression by pH stress it could have been masked by the loss activity caused by the instability of the enzyme (Flint, 1974).

6.2.5 The effect of heat shock on Alkaline Phosphatase Activity of *Escherichia coli*.

It is generally accepted that heat shock of *E.coli* induces the synthesis of some novel proteins and represses the synthesis of some others. The aim of this experiment was to determine whether or not alkaline phosphatase synthesis was also affected by heat shock of starvation-stressed cells. Alkaline phosphatase activity was measured in *E.coli* kept at 54°C for 13 d. The results are shown in Fig 6.12. Alkaline phosphatase activity declined by about 35% in the first 24 hours at 54°C but remained constant for the next 3 d before declining gradually and becoming undetectable after 13 d. Viable counts indicated that the cells lost viability steadily over the first 24 h and were undetectable after 3 d. Again although the viable count disappears either cells are becoming stressed and unable to grow on nutrient agar and activity is retained within the cells. Alternatively alkaline phosphatase is more stable than the cells with respect to heat shock. Alkaline phosphatase is known to be stable to heat over a short time period (Flint, 1974) but its stability at 54°C over this length of time has not previously been reported.

Figure 6.11 The effects of pH stress on alkaline phosphatase activity of *Escherichia coli* in filtered-autoclaved lake water.

E. coli was grown in a low phosphate minimal medium overnight at 30°C. The cells were harvested, washed and resuspended in sterile lake water. 100 ml filtered-autoclaved lake water microcosms were inoculated to give an initial viable count of 10^6 cfu/ml. The pH of the microcosms was adjusted to 5.5, 6.0, 6.5, 7.0 and 9.0 with pH meter. The flasks were incubated in the dark without shaking at 15°C. Alkaline phosphatase activity was measured by removing aliquots, buffering the pH 9.0 with Tris-buffer, and using pNPP as a colorimetric substrate. Activity assays were performed at 30°C. Viable counts were determined on a surface spread plates after overnight incubation at 37°C. Total alkaline phosphatase activity is expressed as change in absorbance at 420 nm/3 h. These can be converted to μ M pNP released/ml/h by multiplying by 15.7.

Figure 6.11



6.2.6 The effect of oxidation stress on alkaline phosphatase activity of *Escherichia coli*.

Several enzymes have been identified as being induced or derepressed under a variety of oxidative stress conditions (Farr and Kogoma, 1991). Here ethanol and hydrogen peroxide caused derepression of the synthesis of alkaline phosphatase in *E. coli* grown in nutrient broth or minimal medium with a high phosphate concentration. In these media there is usually little or no detectable alkaline phosphatase activity due to the high phosphate concentration repressing synthesis of alkaline phosphatase. Table 6.3 shows that after the addition of hydrogen peroxide at 18 mM or ethanol at 217 mM final concentrations to exponentially growing *E. coli* there was derepression of alkaline phosphatase. Alkaline phosphatase activity increased between 13.6 and 41.5 times the control, a greater increase than is often seen due to derepression of enzyme synthesis by phosphate limitation. However, the addition of hydrogen peroxide to filtered-autoclaved lake water led to no increase in alkaline phosphatase activity (Fig 6.13). The difference between the cells in filtered-autoclaved lake water and nutrient broth was the growth phase. It is possible that the cells have to be in exponential phase to respond to oxidative stress.

Cells which had been induced by the addition of hydrogen peroxide to minimal medium showed no further increase in activity upon addition to filtered-autoclaved lake water and the activity declined over an 8 d incubation period at 15°C. The viable count of cells stressed with hydrogen peroxide in filtered-autoclaved lake water or in nutrient broth declined in numbers over the 8 d incubation period whereas the viable count in the control remained constant. Again loss in numbers was not matched by a loss in alkaline phosphatase activity.

Cells shocked with ethanol showed similar results to *E. coli* treated with hydrogen peroxide.

Figure 6.12 The effects of heat shock on alkaline phosphatase activity of *Escherichia coli*.

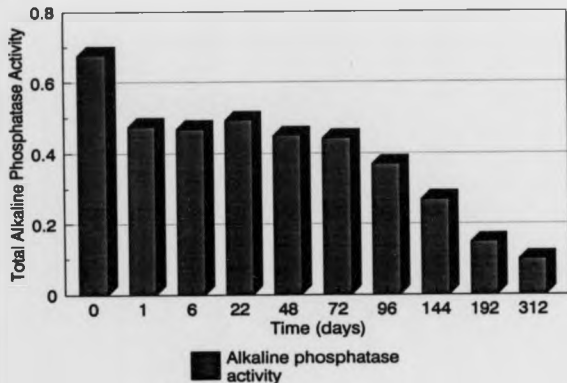
Figure 6.12a The effect of continuous high temperature on alkaline phosphatase activity of *Escherichia coli*.

E. coli was grown in a low phosphate minimal medium overnight at 30°C. The cells were harvested, washed and resuspended in sterile lake water. 100 ml filtered-autoclaved lake water microcosms were inoculated to give an initial viable count of 10^6 cfu/ml. The flasks were kept at 54°C for 13 d dark without shaking at 15°C. Alkaline phosphatase activity was measured by removing aliquots, buffering the pH 9.0 with Tris-buffer, and using pNPP as a colorimetric substrate. Activity assays were performed at 30°C. Viable counts were determined on a surface spread plates after overnight incubation at 37°C. Total direct count was carried out under epifluorescence microscopy by via acridine orange staining. Total alkaline phosphatase activity is expressed as change in absorbance at 420 nm/3 h. These can be converted to μ M pNP released/ml/h by multiplying by 15.7.

Figure 6.12b The effect of preheating on alkaline phosphatase activity of *Escherichia coli*.

E. coli was grown in a low phosphate minimal medium overnight at 30°C. The cells were harvested, washed and resuspended in sterile lake water. 100 ml filtered-autoclaved lake water microcosms were inoculated to give an initial viable count of 10^6 cfu/ml. Beforehand cells were kept at 54°C for 10 minutes in purpose of heat shock then the flasks were incubated in the dark without shaking at 15°C. Alkaline phosphatase activity was measured by removing aliquots, buffering the pH 9.0 with Tris-buffer, and using pNPP as a colorimetric substrate. Activity assays were performed at 30°C. Viable counts were determined on a surface spread plates after overnight incubation at 37°C.

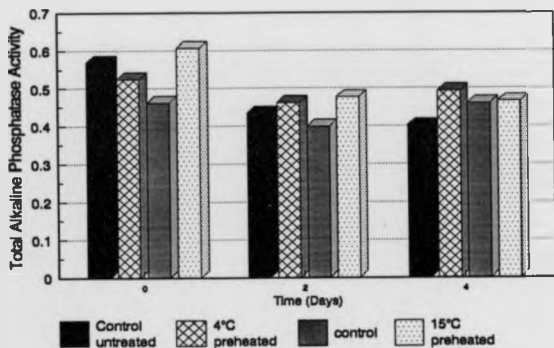
Figure 6.12a



Log viable count

Time (hours)	(cfu/ml)	Log TDC (per/ml)
0	6.0	6.85
2	none	6.79
6	-	7.0
22	-	6.98
48	-	6.94
72	-	6.82
96	-	6.82
144	-	7.0
192	-	6.95
312	-	7.0

Figure 6.12b



Log viable count (cfu/ml)

Time (days)	control	4°C	Control	15°C
0	5.07	5.0	5.1	5.0
2	5.64	none	5.58	none
4	5	none	5.92	none

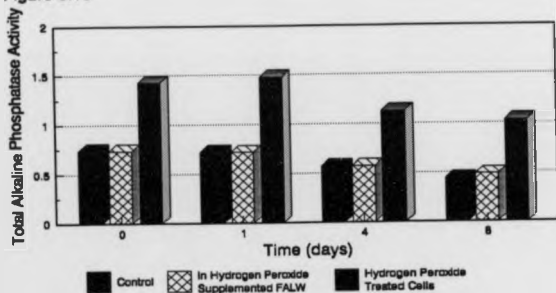
6.2.7 The effect of chlorine stress on alkaline phosphatase activity of *Escherichia coli*.

Disinfectants added to water as part of the water purification process result in a portion of the microbial population becoming injured due to sublethal lesions. The injury process can cause a significant underestimation of indicator organisms. Chlorine stress could cause similar physiological changes in bacteria as the other stresses studied in this chapter therefore changes in alkaline phosphatase activity in chlorine-stressed organisms was investigated. Chlorine stress was induced by the addition of sodium hypochlorite to filtered-autoclaved lake water and incubating the cells at 15°C for a period of 22 d. The results are presented in Fig 6.14. There was no derepression of enzyme synthesis after stress was imposed but the activity remained higher in the samples exposed to sodium hypochlorite than in the unexposed control. Activity was up to 50% higher after 22 d incubation in the presence of 0.15 M and 0.145 M sodium hypochlorite and 33% higher in those exposed to 0.29 M sodium hypochlorite. Although activity remained high, the viable count declined in the stressed samples over the incubation period.

Figure 6.13 The effect of oxidation stress on alkaline phosphatase activity of *Escherichia coli*.

E. coli was grown in a low phosphate minimal medium overnight at 30°C. The cells were harvested, washed and resuspended in sterile lake water. 100 ml filtered-autoclaved lake water microcosms were inoculated to give an initial viable count of 10^7 cfu/ml. Cells were stressed by the addition of 18 mM hydrogen peroxide. Cells were also grown in nutrient broth to which 18 mM hydrogen peroxide was added when the cells were in exponential phase. When stationary phase was reached, cells were harvested and treated as above. The flasks were incubated in the dark without shaking at 15°C. Alkaline phosphatase activity was measured by removing aliquots, buffering the pH 9.0 with Tris-buffer, and using pNPP as a colorimetric substrate. Activity assays were performed at 30°C. Viable counts were determined on a surface spread plates after overnight incubation at 37°C. Total alkaline phosphatase activity is expressed as change in absorbance at 420 nm/3 h. These can be converted to μM pNP released/ml/h by multiplying by 15.7.

Figure 6.13



Log viable count (cfu/ml)

Time (days)	control	Hyd.per.amended	Hyd.per. treated
0	6.97	6.88	6.55
1	7.02	5.0	none
4	6.87	none	
8	6.46		

Table 6.3 The effect of ethanol and hydrogen peroxide on alkaline phosphatase activity of *Escherichia coli*

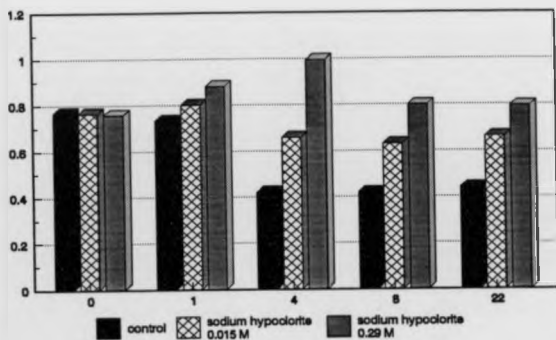
Specific alkaline phosphatase activity				
	In LPMG cells	Ratio	In nutrient broth	Ratio
Control	0.0716	1.0	0.092	1.0
Hydrogen peroxide	1.5500	21.6	1.25	13.6
Ethanol	2.7500	38.4	3.82	41.5

LPMG: Low phosphate medium grown

Figure 6.14 The effect of different concentrations of sodium hypochlorite on alkaline phosphatase activity of *Escherichia coli* at 15°C.

E. coli was grown in a low phosphate minimal medium overnight at 30°C. The cells were harvested, washed and resuspended in sterile lake water. 100 ml filtered-autoclaved lake water microcosms were inoculated to give an initial viable count of 10^7 cfu/ml. The flasks were stressed by the addition of sodium hypochlorite to give final concentrations of 0.015 M, 0.145 M and 0.29 M. The flasks were incubated in the dark without shaking at 15°C. Alkaline phosphatase activity was measured by removing aliquots, buffering the pH 9.0 with Tris-buffer, and using pNPP as a colorimetric substrate. Activity assays were performed at 30°C. Viable counts were determined on a surface spread plates after overnight incubation at 37°C. Total alkaline phosphatase activity is expressed as change in absorbance at 420 nm/3 h. These can be converted to μM pNP released/ml/h by multiplying by 15.7.

Figure 8.14



Log viable count (cfu/ml)

Time (days)	control	0.015 M	0.29
0	7.0	7.08	7.02
1	7.0	6.91	6.31
4	6.54	6.44	4.9
8	5.94	5.98	3.47
22	6.28	6.3	none

6.3 Discussion

The enzyme activity of bacterial cells may have an important function in the survival of bacteria under different stress conditions. The ability of a cell to synthesise protective proteins or to repair damage through enzyme activity is obviously of importance to a stressed cell. Some catabolic enzymes show alterations in activity in bacteria subjected to changing environmental conditions especially stress conditions such as osmotic stress, starvation stress, oxidation stress or heat shock. Alkaline phosphatase is one of the important enzymes in cell metabolism. It is induced in the absence of inorganic phosphate in the growth medium. The enzymes are therefore produced in response to a form of starvation stress, i.e. the unavailability of a specific growth nutrient. There have been suggestions that the enzyme may be produced in response to other forms of stress notably osmotic shock. Consequently the enzyme may be involved in the survival of bacteria under adverse conditions possibly through its action as a scavenger for phosphate. Alternatively the response may be due to the alterations in the bacterial membranes produced by the porin proteins synthesised along with alkaline phosphatase.

In this section alkaline phosphatase activity was measured to determine the effects of long-term starvation stress, osmolarity, heat shock and oxidation stress on enzyme activity in *E. coli* in filtered-autoclaved lake water. All of these factors can affect the survival of *E. coli* in filtered-autoclaved lake water microcosms.

Alkaline phosphatase activity was increased by starvation in microcosms for 70 d at a range of temperature from 4°C to 37°C. Similar results were obtained by Gauthier *et al.* (1990) who showed that alkaline phosphatase activity increased in *E. coli* starved in nutrient-free seawater. They speculated that this could be an adaptation to starvation conditions. It could be argued that it is not a surprise that alkaline phosphatase activity increased under these conditions. The

cells are phosphate limited i.e. they meet the criterion necessary for derepression of alkaline phosphatase in the laboratory. However derepression in the laboratory requires that the cells are growing. Here derepression occurred with no increase in cell numbers i.e. the cells were not growing and at 4°C a temperature at which protein synthesis in *E.coli* is not thought to be carried out. At 37°C the viable count of *E.coli* in the microcosm fell below the detection limit although alkaline phosphatase was still measurable. This could be due to the stability of the enzyme even though the cells are dead or due to the cells becoming viable but non-culturable. There is no evidence from these experiments that *E.coli* actually synthesise alkaline phosphatase continuously. In comparable experiment carried out in distilled water, a truly nutrient-limited environment, enzyme activity still increased in *E.coli* although the viable count fell more dramatically at 30°C and 37°C. The distilled water results suggest that at temperatures at which protein synthesis is more likely to still be carried out the increase in alkaline phosphatase activity is highest.

As shown in Chapter 4 some nutrient amendments, mainly amino acids, ammonium salts and carbon sources, increased the survival times of *E.coli* in filtered-autoclaved lake water microcosms. These same nutrient sources were used to investigate their effect on alkaline phosphatase activity during the survival of *E.coli* in filtered-autoclaved lake water. The results suggested that derepression of alkaline phosphatase activity could occur in filtered-autoclaved lake water after the addition of some amino acids and carbon sources. If *E.coli* grown in high phosphate medium was added to filtered-autoclaved lake water then alkaline phosphatase activity became detectable. However, ammonium sulphate and ammonium nitrate which had the most noticeable effect on the survival of *E.coli* in filtered-autoclaved lake water had no effect on alkaline phosphatase activity. It is generally accepted that alkaline phosphatase synthesis is derepressed in growth media. Here derepression occurred in starvation medium in which little if any growth could occur. Glutamate and proline caused

most derepression. These were also the same amino acids which had most effect on survival (Lim and Flint, 1989). If *E. coli* which already had a derepressed enzyme was added to the filtered-autoclaved lake water microcosms amended with amino acids then some amino acids such as glutamate, and glycine, caused alkaline phosphatase activity to increase further. The effects of glutamate on alkaline phosphatase activity were related to concentration added suggesting that it was related to the ability of this amino acids to maintain viability or at least maintain the metabolic capability of the cell which was involved in its survival. The increase in alkaline phosphatase activity could enable *E. coli* cells to cope with changing environmental conditions through the production of more enzyme and the proteins associated with the *pho* operon. The increase in activity could simply be a reflection of the low phosphate regime of the filtered-autoclaved lake water which leads to derepression when there is a carbon source such as an amino acid available to meet the energy requirement of the cell. The interesting phenomenon here is that activity is retained or increased even without any increase in bacterial viable count and in some cases when the viable count is falling.

Carbon sources such as carbohydrates and carboxylic acids also affected the alkaline phosphatase activity of *E. coli* in filtered-autoclaved lake water. Gadkari (1984) has shown that the addition of glucose plus nitrogen sources to soil increased alkaline phosphatase activity. This may be because alkaline phosphatase is required for catabolic reaction which need energy therefore the increase in activity seen with some carbon sources could be a reflection of this requirement (Strayer, 1981). Glycerol and the acetate had most effect but most of the carbon sources used increased alkaline phosphatase activity in the microcosms. Again derepression could have simply been a reflection of the phosphate limited nature of the growth medium once a carbon source had been added. However the enzyme was once again derepressed and changes in enzyme activity occurred in microcosms where the viable count was static or decreasing.

This shows once again that synthesis of this enzyme was not linked to growth but rather to survival of the *E. coli* cells.

Osmotic stress caused by increasing concentrations of sodium chloride is an effective inducer of general stress proteins (Hecker *et al.*, 1988). Osmotic stress is also an important environmental factor especially to *E. coli* discharged directly in to the sea or entering the marine environment from rivers. Changes in the osmolarity of a change in alkaline phosphatase activity (Villarejo *et al.*, 1983). *E. coli* responds to changes in osmolarity by changing several properties or uptake of osmoprotectants, such as proline (Imhoff and Rodriguez-Valera, 1984; LeRudulier *et al.*, 1984a); the internal K^+ concentration (Epstein, 1986); the amount of oligosaccharide in the periplasmic space (Kennedy, 1982); and the relative amount of the outer membrane proteins, OmpF and OmpC (Kawaji *et al.*, 1979). *E. coli* exposed to osmotic stress in filtered-autoclaved lake water showed an increase in alkaline phosphatase activity. The increase in alkaline phosphatase activity in *E. coli* grown in low phosphate medium was proportional to the increase in salt concentration. There was no increase in activity in cells grown in high phosphate medium. This suggested that osmolarity did not cause derepression of alkaline phosphatase but led to further synthesis of the enzyme if this was already derepressed. Alternatively the increase could be an artefact caused by the osmolarity affecting the outer permeability of the *E. coli* thus allowing easier access for the substrate to the periplasmic space where the enzyme is located. Easier permeability would be shown by an increase in activity. The addition of osmoprotectants also caused a reduction in any increase in alkaline phosphatase activity suggesting that the action of increased osmolarity was inside the cell rather than an artefact of permeability of the outer membrane. This would still have been affected by the osmolarity of the external medium even in the presence of osmoprotectants which function inside the cell.

Another important factor which could determine the survival of bacteria in the aquatic environment is the ambient pH. Alkaline phosphatase has an

optimum pH of around 9.0 both in organisms studied in the laboratory (Torriani, 1968) and in natural samples (Reichardt *et al.*, 1967; Matavulji and Flint, 1987). As the pH of lake water can change seasonally the survival of *E. coli* and the alkaline phosphatase activity of cells starved in filtered-autoclaved lake water was monitored. The final alkaline phosphatase activity of *E. coli* kept at alkaline pH was higher than that of cells starved at acidic pH. This is more likely to be a reflection of the instability of alkaline phosphatase at acidic pH changes on the synthesis of the enzyme (Flint and Hopton, 1976).

All organisms produce novel proteins in response to heat shock treatment. Woodcock and Grigg (1972) reported that heat shock also causes breaks in single and double stranded DNA. Other changes to nucleic acids and the leakage of internal components through damaged membranes have been reported (Iandolo and Ordal, 1966; Welker, 1976). The damage to membranes also has effects on the efficiency of nutrient transport and hence it might be expected that membrane damage would have an effect on the survival of bacteria under starvation conditions (Gray, 1978). Here *E. coli* was kept at 54°C for a period of 13 d in filtered-autoclaved lake water. Alkaline phosphatase activity gradually declined over this time period but the viable count declined very quickly. Again there was no evidence that new enzyme was being produced as the viable count declined rather it is more likely that the residual enzyme represents performed enzyme which is more resistant to heat treatment than are the bacterial cells. Although a number of the proteins produced in response to starvation stress and heat shock are similar, it does not appear that alkaline phosphatase or other proteins of Pho regulon are synthesised in response to heat shock. *E. coli* grown in high or low phosphate minimal medium and subjected to 10 minutes of heat shock showed no derepression of enzyme synthesis over a further 13 d starvation period in filtered-autoclaved lake water at 15°C.

Several enzymes have been shown to be induced by oxidation stress (Farr and Kogoma, 1991). Christman *et al.* (1985) have shown that some general

stress proteins are also produced at different times during stress caused by hydrogen peroxide. Here alkaline phosphatase activity was increased by up to 41 times in the presence of hydrogen peroxide or ethanol in growth medium which contained a high phosphate concentration (usually this concentration would repress the synthesis of alkaline phosphatase). This suggest that alkaline phosphatase is one of the enzymes which can be induced by oxidation stress and is part of the stress response in *E.coli*. However, stressing the cells in the starvation medium of filtered-autoclaved lake water did not lead to any derepression of enzyme activity. Again alkaline phosphatase activity was measurable for many days after the viable count had become undetectable. Similar stress caused by sodium hypochlorite also increased alkaline phosphatase activity above that of unexposed controls. Stress caused by the chlorination of water samples is known to make a large proportion of the population undetectable, however these cells can recover if they are given a sufficient nutrient supply (LeChevallier *et al.*, 1985; Camper and McFeters 1979). It might be expected that part of this response to the need for nutrients to aid recovery could be the derepression of a scavenging enzyme such as alkaline phosphatase. Enzyme activity was again detectable for a longer period than the viable count of stressed cells.

Overall alkaline phosphatase activity of *E.coli* was derepressed by stress including starvation stress. The addition of nutrients to filtered-autoclaved lake water also led to an increase in alkaline phosphatase activity but this could be a reflection of the attempts by cells to grow on the added nutrients in what is now a phosphate-limited medium. Although there was rarely any increase in viable count enzymes could still be derepressed or induced under these conditions. In some instances cells grown in high phosphate medium showed derepression upon addition to filtered-autoclaved lake water amended with nutrients. Again this suggests that *E.coli* was able to grow in the filtered-autoclaved lake water medium and synthesise this enzyme under these conditions.

Chapter VII. β -Galactosidase activity of *Escherichia coli* under diferent nutrient conditions in lake water.

7.1 Introduction

As shown with other bacteria, *E.coli* also undergoes some physiological modifications such as enzyme activity (Munro *et al.*, 1987), protein synthesis (Reeve, 1984), and changes to cell envelopes (Zanke *et al.*, 1980) under nutrient deprivation and different growing conditions. Such cellular alterations under starvation stress and excess nutrient conditions could enable microorganisms to adapt to survive in the surrounding environment (Morita, 1985). There is a possibility that β -galactosidase enzyme activity of *E.coli* may be affected by environmental changes for instance the lack of nutrients, or the presence of some nutrients in the aquatic environment. This may also be an important factor in terms of human health aspect. Many surface waters are monitored for the presence of total coliforms and faecal coliforms by means of a β -galactosidase assay which has been developed as one of the faster detection methods e.g. the Autoanalysis Colilert method. (Edberg *et al.*, 1990; Manafi *et al.*, 1991). This detection method is based on the utilisation of fluorogenic and chromogenic substrates. The enzymatic hydrolysis of the substrate ONPG is carried out by β -galactosidase in faecal coliforms (Warren *et al.*, 1978; Edberg *et al.*, 1991). Therefore the effects of different factors in lake water on the induction of β -galactosidase activity is important.

β -Galactosidase is a catabolic enzyme (EC.3.2.1.23). It cleaves lactose into galactose and glucose (Edberg *et al.*, 1990). This enzyme is a typical cytosolic periplasmic protein which is released to the cell membrane but not to periplasm (Neidhardt *et al.*, 1990). The importance of this enzyme in terms of detection was suggested by Warren *et al.* (1978). There was a linear correlation between faecal coliform most probable number values and o-nitrophenyl- β -D galactoside (ONPG) hydrolysis times. In addition, Munro *et al.* (1987) speculated that the disappearance of β -galactosidase activity in *E.coli* cells in

seawater could have significant implications for their enumeration by standard culture method.

As it has been reported in chapter 5 and 6, the activity of alkaline phosphatase and respiratory dehydrogenase activity were altered in filtered-autoclaved lake water by the effect of nutrient limitation and the addition of carbon and nitrogen sources. Others have reported that β -galactosidase activity was also influenced by the nutritional status of the medium. Berg and Fiksdal (1988) suggested that the enzymes activity is subjected to the physiological status of bacteria and 10 to 90% of coliforms may be stressed. Anderson *et al.* (1979) showed that β -galactosidase activity of *E. coli* was reduced by increasing starvation time and became undetectable after 13 days of incubation. Also Munro *et al.* (1987) showed that β -galactosidase activity of *E. coli* diminished gradually with incubation period even though some other enzymatic activities such as alkaline phosphatase activity increased. Furthermore McLeod *et al.* (1975) have reported that the highest β -galactosidase activity occurred under succinate and glycerol limitation. Pine (1980) reported that there was no β -galactosidase activity under histidine starvation. Besides, temperature might be another important factor affecting *E. coli* β -galactosidase activity in lake water. Pine (1980) reported that β -galactosidase synthesis in *rel A*⁻ mutant *E. coli* cells was more sensitive to temperature and had a lower activity than normal cells.

Manafi *et al.* (1991) suggested that prior growing conditions and medium composition gave different β -galactosidase activity in the cells. Warren *et al.* (1978) noted that the activity of β -galactosidase was higher in samples collected from water containing high organic and inorganic nutrients. In the concept of bacterial economy, microorganisms do not synthesise all enzymes unless they are necessary for metabolic reactions under current physiological conditions (Gottschalk, 1986). Induction of β -galactosidase activity of *E. coli* may be a big burden for the survival of *E. coli* since it has been shown that mRNA appeared and disappeared quickly during starvation. The quantity of specific messenger

present in the cells must be proportional to the synthesis of β -galactosidase enzyme (Neidhart *et al.*, 1990).

There have been no previous studies on β -galactosidase activity of *E.coli* under starvation stress in aquatic environment. The aim of study was to investigate a possible relationship between starvation, survival and β -galactosidase activity of *E.coli*. β -galactosidase activity may reflect the metabolic activity changes of *E.coli* cell under different nutritional conditions at different temperatures and provide more knowledge about the factors affecting the survival and detection of *E.coli* in lake water microcosms.

7.2 Result

7.2.1 β -galactosidase activity of *Escherichia coli* in filtered-autoclaved lake water at different temperatures.

The results show that β -galactosidase activity of *E.coli* remained relatively constant for 60 days in filtered-autoclaved lake water without any nutrient amendments at 4°C and 15°C (Figure 7.1a). However the activity of β -galactosidase declined rapidly in the cells incubated at 37°C with more than 80% of activity being lost in the first 24 h. The reduction in activity was also faster at 25°C than 4°C and 15°C with 80% of activity lost in 7 days. This sudden decline in β -galactosidase activity in *E.coli* showed similarity to the loss of respiratory enzymes under starvation stress at 37°C (Chapter 5). The loss of viability of *E.coli* took place after 7 days of incubation in filtered-autoclaved lake water at 37°C (Figure 7.1b). Although the activity of β -galactosidase enzyme in *E.coli* cells began to decline after 3 days of incubation, there was not a decline in viability of the cells in filtered-autoclaved lake water at 25°C until after 15-17 days. The level of β -galactosidase activity of *E.coli* at different temperatures was the highest at 15°C compared to other incubation temperatures, even though the samples had the same initial inoculum size and remained relatively constant for up to 60 days. β -galactosidase activity was increased by 60% after 60 days at 4°C and 20% at 15°C after 5 days. There was no decline in the viable count of *E.coli* cells at either temperature. These results suggest that metabolic changes occur in *E.coli* cells under starvation stress at 25°C and 37°C in filtered-autoclaved lake water. These changes may be indicated by the activity of β -galactosidase enzyme activity under certain circumstances. It could be argued that β -galactosidase activity is lost in response to starvation stress at 25°C and 37°C

Figure 7.1 β -Galactosidase activity of *Escherichia coli* at different temperatures in filtered-autoclaved lake water.

7.1a. *E. coli* was grown in basal medium containing glycerol as a sole of carbon and energy source overnight at 30°C. IPTG (isopropylthio-galactoside) was added to the minimal medium as an inducer of β -galactosidase. The cells were harvested and washed with 0.05 M sodium phosphate buffer (pH 7.2) and resuspended in sterile lake water. 100 ml sterile lake water microcosms were inoculated to give an initial viable count of approximately 10^7 cfu/ml. The flasks were incubated in the dark without shaking at 4°C, 15°C, 25°C and 30°C. β -Galactosidase activity was measured by adding 1 ml aliquots to 4 ml 0.05 M sodium phosphate. 0.5 ml of 0.01 M ONPG as a colorimetric substrate was used. Activity assays were carried out at 30°C for 4 h. Activity is expressed as change in absorbance at 420 nm/4 h. Viable counts were determined on surface spread plates after overnight incubation at 37°C (7.1b).

Fig.7.1a

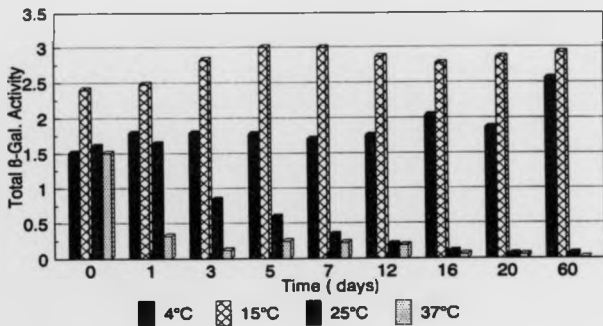
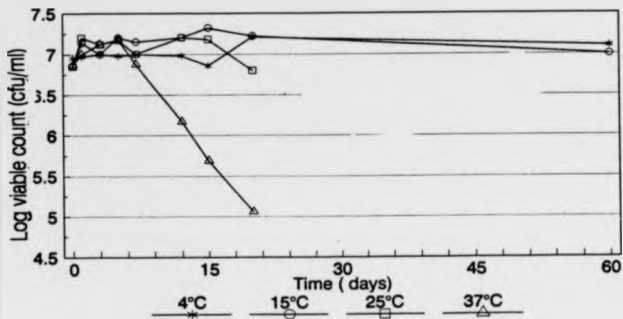


Fig.7.1b



as *E.coli* utilises unessential proteins. Once there have been consumed then *E.coli* loses viability.

7.2.2 β -Galactosidase activity of *Escherichia coli* in filtered-autoclaved lake water amended with nutrient sources.

In these experiments, β -galactosidase activity of *E.coli* was followed after the addition of single carbon and nitrogen sources to filtered-autoclaved lake water. Different incubation temperatures were used to study effects of these changes on enzyme activity was observed. The aim of these experiments was to investigate the link between β -galactosidase activity and survival after the addition of nutrient sources to lake water.

7.2.2.1 β -galactosidase activity of *Escherichia coli* in filtered-autoclaved lake water amended with carbon sources.

Figure 7.2 shows that the addition of carbon sources had little effect on the specific activity with all samples showing a gradual decline. None of the carbon sources led to an increase in β -galactosidase specific activity. Glycerol, lactose and sucrose caused a reduction in β -galactosidase activity after 16 days of incubation at 15°C. Total activity in these samples remained relatively constant as in the previous experiments (Fig. 7.1). The decline in specific activity was due to an increase viable count without an increase in β -galactosidase activity. This suggests that the new cells do not synthesise new enzyme even in the presence of lactose as an inducer.

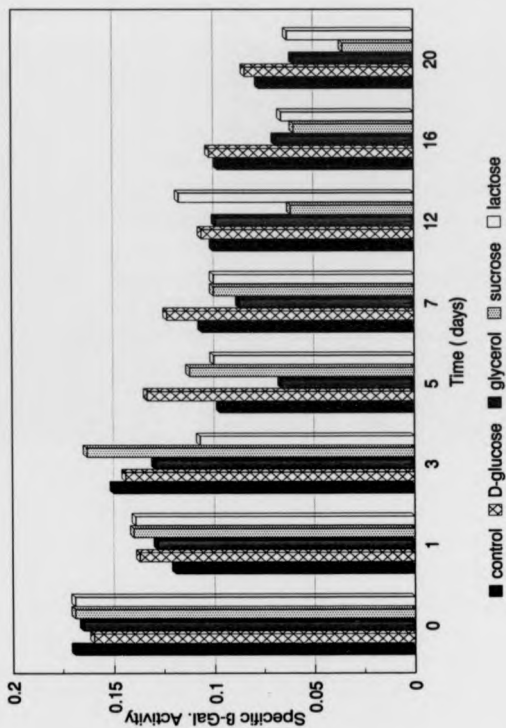
7.2.2.2 β -Galactosidase activity of *Escherichia coli* in filtered-autoclaved lake water amended with amino acids.

The addition of some amino acids to filtered-autoclaved lake water led to

Figure 7.2 β -Galactosidase activity of *Escherichia coli* in filtered-autoclaved lake water amended with carbon sources.

E. coli was grown in basal medium containing glycerol as a sole of carbon and energy source overnight at 30°C. IPTG was added to the minimal medium as an inducer of β -galactosidase. The cells were harvested, washed with 0.05 M sodium phosphate buffer (pH 7.2) and resuspended in sterile lake water. 100 ml sterile lake water microcosms were individually amended with 4 mg-C/L of glucose, glycerol, sucrose and lactose then inoculated to give an initial viable count of approximately 10^7 cfu/ml. The flasks were incubated in the dark at 15°C. β -Galactosidase activity was measured by removing adding 1 ml aliquots to 4 ml 0.05 M sodium phosphate buffer (pH 7.2). 0.5 ml of 0.01 M ONPG was added as colorimetric substrate. Activity assays were carried out at 30°C. Viable counts were determined on surface spread plates after overnight incubation at 37°C. Specific activity is expressed as change in absorbance at 420 nm/4 h/cfu.

Fig.7.2



an increase in both the total and the specific activity of β -galactosidase at 4°C, 15°C and 30°C (Table 7.1-7.3 and Fig 7.3-7.5). Some amino acids however had little effect on activity. Fig 7.4 and 7.5 show that the specific activity was significantly enhanced at 15°C and 30°C after the addition of glycine and L-methionine. In each case the increase in specific activity was proportional to the concentration of the amino acids added. In each case it was an increase in total activity which led to the increase in specific activity rather than a decrease in viable count which would have the same effect. This suggests that there is further induction of β -galactosidase under starvation conditions even in the absence of IPTG, the accepted inducer of activity. Even at 4°C there was an increase in total activity. As protein synthesis was considered unlikely to be occurring in *E.coli* at 4°C this increase was probably due to permeability changes in the organisms induced by the reduction in temperature or starvation, or both.

An increase in specific activity was also seen with additions of proline and serine. Once again there was some indication that the increase was linked to the concentration of the amino acid added. It was surprising that again the total activity was higher at 4°C than at 15°C or 30°C. The changes to the specific activity of the enzyme were often apparent within 24 h of addition of the amino acids. After 7 days or more than increase was often four-fold or more, an increase not explainable by simple permeability changes but due to real increase in enzyme activity.

7.2.2.3 β -Galactosidase activity of *Escherichia coli* in filtered-autoclaved lake water amended with ammonium sulphate and ammonium nitrate.

In chapter 4 it was shown that the disappearance of *E.coli* in the presence of the natural microflora was delayed by the addition of ammonium sulphate and ammonium nitrate to untreated lake water. Therefore the effects of ammonium

sulphate and ammonium nitrate on β -galactosidase were examined at 4°C, 15°C and 30°C in filtered-autoclaved lake water. Figure 7.6 shows that the specific activity of β -galactosidase activity of *E. coli* was increased by the addition of 10, 20 and 30 mg/L ammonium sulphate to lake water at 15°C. Table 7.1 and 7.3 show that a similar increase occurred at 4°C and 30°C respectively. The rate of increase was proportional to ammonium sulphate concentrations added at 4°C and 30°C but this relationship was conclusive at 15°C. Similar results were obtained after the addition of ammonium nitrate at 4°C, 15°C and 30°C (Fig 7.7a,b; Table 7.1-7.3). Again the increase was due to an increase in total activity suggesting that either cells were more permeable or that there was an increase in the production of β -galactosidase.

Figure 7.3 β -Galactosidase activity of *Escherichia coli* in filtered-autoclaved lake water amended with amino acids.

E. coli was grown in basal medium containing glycerol as a sole of carbon and energy source overnight at 30°C. IPTG was added to the minimal medium as an inducer of β -galactosidase. The cells were harvested, washed with 0.05 M sodium phosphate buffer (pH 7.2) and resuspended in sterile lake water. 100 ml sterile lake water microcosms were individually amended with 4 mg/L L-proline, 4 mg/L L-serine, 4 mg/L L-arginine, 4 mg/L L-alanine and inoculated to give an initial viable count of approximately 10^7 cfu/ml. The flasks were incubated in the dark at 15°C. β -Galactosidase activity was measured by removing adding 1 ml aliquots to 4 ml 0.05 M sodium phosphate buffer (pH 7.2). 0.5 ml of 0.01 M ONPG was added as colorimetric substrate. Activity assays were carried out at 30°C. Viable counts were determined on surface spread plates after overnight incubation at 37°C. Specific activity is expressed as change in absorbance at 420 nm/4 h/cfu.

Fig.7.3

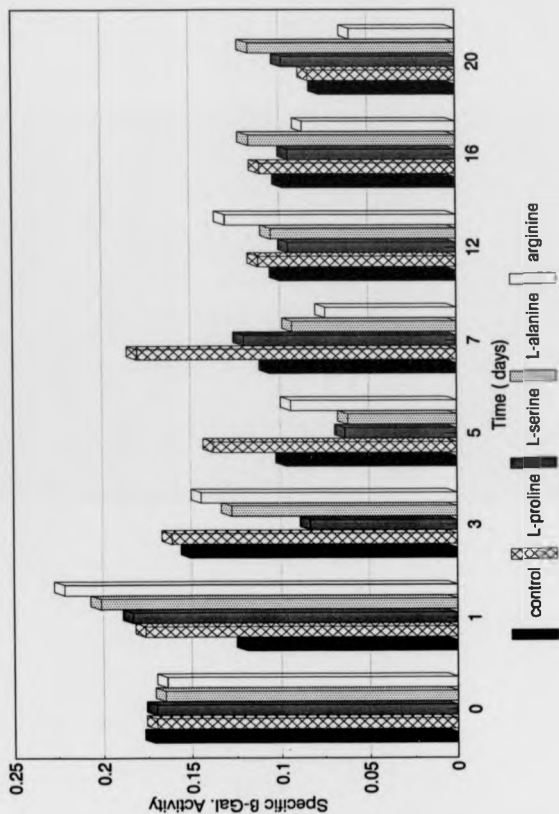


Figure 7.4 β -Galactosidase activity of *Escherichia coli* in filtered-autoclaved lake water amended with different concentration of glycine.

E. coli was grown in basal medium containing glycerol as a sole of carbon and energy source overnight at 30°C. IPTG was added to the minimal medium as an inducer of β -galactosidase. The cells were harvested and washed with 0.05 M sodium phosphate buffer (pH 7.2) and resuspended in sterile lake water. 100 ml sterile lake water microcosms were individually amended with 0.5 mg/L, 1 mg/L and 1.5 mg/L of glycine then inoculated to give an initial viable count of approximately 10^7 cfu/ml. The flasks were incubated in the dark without shaking at 15°C (Fig 7.4a) and 30°C (Fig 7.4b). β -Galactosidase activity was measured by adding buffering 1 ml aliquots to 4 ml 0.05 M sodium phosphate buffer (pH 7.2). 0.5 ml of 0.01 M ONPG was used as a colorimetric substrate. Activity assays were carried out at 30°C. Viable counts were determined on surface spread plates after overnight incubation at 37°C. Activity is expressed as change in absorbance at 420 nm/4 h/cfu.

Fig.7.4a

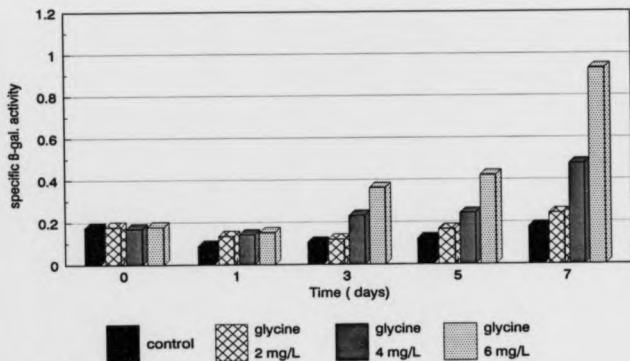


Fig.7.4b

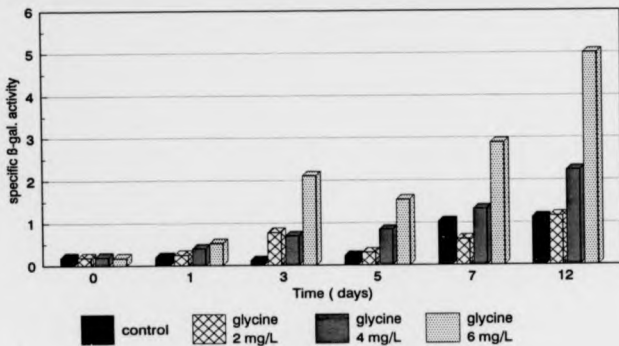


Figure 7.5 β -galactosidase activity of *Escherichia coli* in filtered-autoclaved lake water amended with L-methionine.

E. coli was grown in basal medium containing glycerol as a sole of carbon and energy source overnight at 30°C. IPTG was added to the minimal medium as an inducer of β -galactosidase. The cells were harvested and washed with 0.05 M sodium phosphate buffer (pH 7.2) and resuspended in sterile lake water. 100 ml sterile lake water microcosms were individually amended with 2 mg/L, 4 mg/L and 6 mg/L of L-methionine then inoculated to give an initial viable count of approximately 10^7 cfu/ml. The flasks were incubated in the dark without shaking 30°C. β -Galactosidase activity was measured by adding buffering 1 ml aliquots to 4 ml 0.05 M sodium phosphate buffer (pH 7.2). 0.5 ml of 0.01 M ONPG was used as a colorimetric substrate. Activity assays were carried out at 30°C. Viable counts were determined on surface spread plates after overnight incubation at 37°C. Activity is expressed as change in absorbance at 420 nm/4 h/cfu.

Fig.7.5

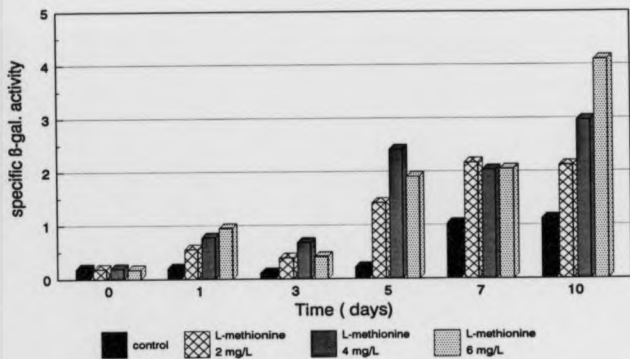


Figure 7.6 β -galactosidase activity of *Escherichia coli* in filtered-autoclaved lake water amended with ammonium sulphate.

E. coli was grown in basal medium containing glycerol as a sole of carbon and energy source overnight at 30°C. IPTG was added to the minimal medium as an inducer of β -galactosidase. The cells were harvested and washed with 0.05 M sodium phosphate buffer (pH 7.2) and resuspended in sterile lake water. 100 ml sterile lake water microcosms were individually amended with 10 mg/L, 20 mg/L and 20 mg/L of ammonium sulphate and inoculated to give an initial viable count of approximately 10^7 cfu/ml. The flasks were incubated in the dark without shaking at 15°C. β -Galactosidase activity was measured by adding buffering 1 ml aliquots to 4 ml 0.05 M sodium phosphate buffer (pH 7.2). 0.5 ml of 0.01 M ONPG was used as a colorimetric substrate. Activity assays were carried out at 30°C. Viable counts were determined on surface spread plates after overnight incubation at 37°C. Activity is expressed as change in absorbance at 420 nm/4 h/cfu.

Fig.7.6

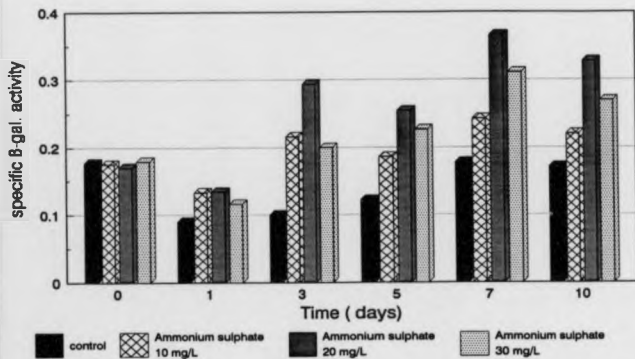


Figure 7.7 β -galactosidase activity of *Escherichia coli* in filtered-autoclaved lake water amended with ammonium nitrate.

E. coli was grown in basal medium containing glycerol as a sole of carbon and energy source overnight at 30°C. IPTG was added to the minimal medium as an inducer of β -galactosidase. The cells were harvested and washed with 0.05 M sodium phosphate buffer (pH 7.2) and resuspended in sterile lake water. 100 ml sterile lake water microcosms were individually amended with 5 mg/L, 10 mg/L and 15 mg/L of ammonium nitrate and inoculated to give an initial viable count of approximately 10^7 cfu/ml. The flasks were incubated in the dark without shaking at 15°C (Fig 7.7a) and at 30°C (Fig 7.7b). β -Galactosidase activity was measured by adding buffering 1 ml aliquots to 4 ml 0.05 M sodium phosphate buffer (pH 7.2). 0.5 ml of 0.01 M ONPG was used as a colorimetric substrate. Activity assays were carried out at 30°C. Viable counts were determined on surface spread plates after overnight incubation at 37°C Activity is expressed as change in absorbance at 420 nm/4 h/cfu.

Fig.7.7a

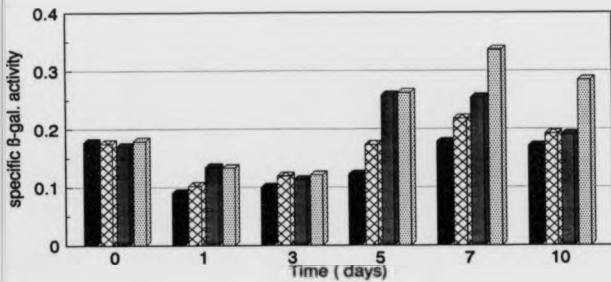


Fig.7.7b

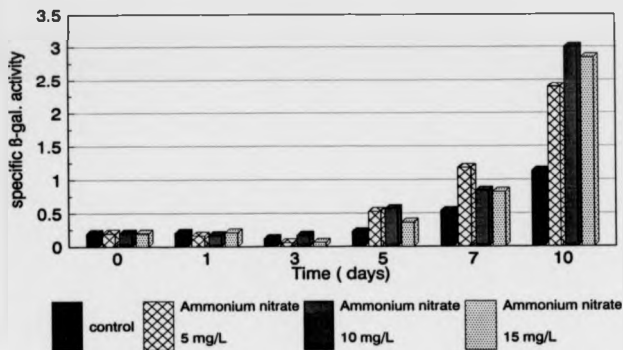


Table 7.1 The effect of nutrient sources on β -galactosidase activity of *Escherichia coli* in filtered autoclaved lake water at 4°C.

nutrient sources concentrations(mg/L)	Total β -Galactosidase activity	Specific β -Galactosidase Activity	Changes in Specific Activity Ratio (compars to control)
Control	0.780	0.11	1.00
Glycine (2.0)	1.500	0.42	3.81
• (4.0)	1.690	4.16	3.76
• (6.0)	1.140	0.36	3.27
L-proline(2.0)	1.060	0.18	1.63
• (4.0)	0.970	0.13	1.18
• (6.0)	0.750	0.18	1.63
Serine (2.0)	1.460	0.27	2.45
• (4.0)	1.120	0.36	3.27
• (6.0)	1.100	0.36	3.27
L-methionine(2.0)	0.850	0.23	2.08
• (4.0)	0.910	0.81	7.36
• (6.0)	0.980	0.35	3.18
Ammonium sulphate(10.0)	1.020	0.27	2.45
• (20.0)	1.140	0.38	3.45
• (30.0)	1.730	0.65	5.90
Ammonium nitrate(5.0)	0.930	0.36	3.27
• (10.0)	0.759	0.18	1.63
• (15.0)	0.800	0.11	1.00

The activity of enzyme was measured after 7 d of incubation.

Table 7.2 The effect of nutrient sources on β -galactosidase activity of *Escherichia coli* in filtered-autoclaved lake water at 15°C.

Nutrient Source Concentrations(mg/L)	Total β -Galactosidase activity	Specific β -Galactosidase Activity	Changes in Specific Activity Ratio (compare to control)
Control	0.69	0.17	1.00
Glycine (2.0)	0.82	0.24	1.41
* (4.0)	0.97	0.47	2.76
* (6.0)	1.11	0.92	5.41
L-proline(2.0)	0.68	0.20	1.17
* (4.0)	0.71	0.22	1.29
* (6.0)	0.71	0.18	1.05
Serine (2.0)	0.68	0.15	0.88
* (4.0)	0.66	0.12	0.70
* (6.0)	0.67	0.21	1.23
L-methionine(2.0)	0.78	0.20	1.17
* (4.0)	0.82	0.17	1.00
* (6.0)	0.85	0.20	1.17
Ammonium sulphate(10.0)	0.86	0.24	1.41
* (20.0)	0.80	0.36	2.11
* (30.0)	0.83	0.31	1.82
Ammonium nitrate(5.0)	0.83	0.21	1.23
* (10.0)	0.78	0.25	1.47
* (15.0)	0.78	0.33	1.94

The activity was measured after 7 d of incubation.

Table 7.3 The effect of nutrient sources on β -galactosidase activity of *Escherichia coli* in filtered-autoclaved lake water at 30°C.

Nutrient source concentrations (mg/L)	Total β -Galactosidase activity	Specific β -Galactosidase Activity	Changes in Specific Activity Ratio (compare to control)
Control	1.09	0.22	1.00
Glycine (2.0)	1.53	0.30	1.36
• (4.0)	1.84	0.84	3.81
• (6.0)	1.82	1.54	7.00
L-proline(2.0)	0.92	0.82	3.72
• (4.0)	1.10	0.13	0.59
• (6.0)	1.13	0.24	1.09
Serine (2.0)	0.65	0.10	0.45
• (4.0)	0.52	0.06	0.27
• (6.0)	1.05	0.87	3.95
L-methionine(2.0)	1.55	1.41	6.40
• (4.0)	1.92	2.42	11.00
• (6.0)	1.75	1.96	8.90
Ammonium sulphate(10.0)	1.39	0.55	2.50
• (20.0)	1.32	1.36	6.18
• (30.0)	1.10	2.76	12.50
Ammonium nitrate(5.0)	1.31	0.52	2.36
• (10.0)	1.52	0.56	2.54
• (15.0)	1.26	0.33	1.50

The activity was measured after 5 d of incubation.

7.3 Discussion

Bacterial enzymes are strongly affected by the organism's nutrition and chemical and physiological environment. As a result, a wide range of changes in physiological activities of bacterial cells may cause some difficulties for the detection of bacteria in environmental samples, especially under starvation stress. Therefore it is important to know what factors affect bacterial enzyme activity and how activity is affected in bacterial cells in natural environments under nutrient limitation. One of the methods has been based on the enzymatic hydrolysis of the substrate o-nitrophenyl- β -D-galactoside (ONPG) by faecal coliforms (Warren *et al.*, 1978). This method has been reported as very sensitive method in terms of accuracy and allows a faster detection of bacteria directly from the environment than traditional culture methods (Warren *et al.*, 1978; Edberg *et al.*, 1989, 1990; Manafi *et al.*, 1991). It has been reported that β -galactosidase activity or the rate of hydrolysis of ONPG is proportional to quantity of faecal coliforms in the inoculum (Warren *et al.*, 1978; Berg and Fiksdal, 1988). Warren *et al.* (1978) suggested that there was a linear correlation between ONPG hydrolysis time and faecal coliform most probable number values. However our results demonstrated here that β -galactosidase activity was not related to the viable count in filtered-autoclaved lake water under starvation stress. *E. coli* still remained viable and culturable after 20 days at 25°C and 37°C, β -galactosidase activity had declined markedly in filtered-autoclaved lake water. This could suggest that long-term starvation in the natural environment may affect the detection of *E. coli* when it is carried out by the detection of β -galactosidase activity. These results suggest that when the metabolic activity of *E. coli* is reduced by starvation β -galactosidase activity is also reduced even though plate counts and alkaline phosphatase activity are not reduced. It is possible that β -galactosidase is a less stable enzyme than alkaline phosphatase. It is also possible that proteolytic activity is directed towards the

less essential enzymes such β -galactosidase rather than those involved in the alleviation of starvation stress such as alkaline phosphatase and other nutrient scavenging enzymes. However, the viability of *E.coli* cells and β -galactosidase activity remained almost constant at 4°C and 15°C during the 60 days experimental period. The increased stability of β -galactosidase activity, which was stable for up to 60 days at 4°C and 15°C, could be explained by decreased proteolytic activity or increased enzyme stability at the lower temperatures.

The results here also suggested that some nutrient sources led to increased β -galactosidase activity of *E.coli* in filtered-autoclaved lake water at different temperatures. It was previously noted by Warren *et al.* (1978) that β -galactosidase activity was increased in the water samples containing high organic and inorganic nutrient concentrations. The data here demonstrated that carbon sources did not have any significant influence on β -galactosidase activity of *E.coli*. However some amino acids had a notable effect on β -galactosidase activity of *E.coli* dependent on incubation temperature. For instance, the proline amendments led to an increase in β -galactosidase activity of at 4°C and 37°C. In addition, although serine did not have any significant influence on β -galactosidase activity in lake water at 15°C and 30°C, it had an effect at 4°C under the same conditions. However, L-methionine showed the strongest effect at 30°C and 15°C but did not cause any changes in activity at 4°C. Furthermore, the effect of some amino acids were link to the concentration with a greater increase in specific activity as the amino acid concentration added to the lake water was increased.

Some other nitrogen sources such as ammonium sulphate and ammonium nitrate also resulted in an increase in β -galactosidase activity in filtered-autoclaved lake water. These nutrient sources are known to be effective nutrient sources in prolonging the survival of *E.coli* in filtered-autoclaved lake water (Lim and Flint, 1989). It has been noted by Neidhardt *et al.* (1990) that the increase in β -galactosidase activity when a inducer added to a growing culture

reflects *de novo* synthesis of enzyme. It is possible that nitrogen sources can be utilised for protein synthesis in *E.coli* cells as a result of which β -galactosidase activity can be increased by the addition of ammonium sulphate and ammonium nitrate to filtered-autoclaved lake water.

In brief, β -galactosidase activity can be affected by different factors in lake water such as nitrogen sources and temperature. Moreover starvation stress can influence β -galactosidase activity, as a consequence, this could lead to problems with the detection and the identification of faecal coliforms. *E.coli* can enter a dormant state in order to survive long-term starvation stress. It is unlikely that β -galactosidase will be synthesised in the *E.coli* cells. Hence the loss of activity in starving cells is a reflection of the denaturation of the enzyme by the proteolytic enzymes. This supplies a nitrogen source for the cells in order for them to continue to survive under nutrient limitation. The increased activity after the amendment of the lake water with a nitrogen source may simply be a reflection of the decreased level of denaturation of existing enzymes which would occur to satisfy the cells nitrogen requirement.

Chapter VIII. Changes in protein patterns of *Escherichia coli* under different stress conditions in lake water

8.1 Introduction

8.1.1 Changes in general protein patterns of *Escherichia coli*

Bacteria need special survival strategies which are essentially important for them to resist changes in an environment e.g. changes in the natural growth-limiting conditions. It has been reported by several microbiologists that the induction of stress proteins is of fundamental importance in this adaptational network. The induction of these proteins occurs in bacteria under steady state conditions which may give the cells a general protection under different stress conditions (Hecker and Völker, 1990). Kjelleberg *et al.* (1987) suggested that bacterial species have more chance to survive in their natural environment by synthesising new proteins when they are faced with starvation stress in natural environments. The starvation specific proteins which are essential for survival of bacteria may be involved in substrate utilisation (Geesey and Morita, 1979), attachment to surface (Kjelleberg *et al.*, 1987), altered chemotaxis (Morita, 1985) or excretion and synthesis of exoproteases (Albertson *et al.*, 1990). The synthesis of *de novo* proteins enables the bacteria to scavenge more efficiently for nutrients under nutrient-limited conditions. This strategy will allow them to overcome stress conditions, eventually confers a more stress resistant phenotype and prolongs survival. This speculation has been supported by the work of Nystöm *et al.* (1990) who showed that pre-starved or cadmium stressed *Vibrio* S14 were more resistant to starvation. The synthesis and degradation of proteins have a role in satisfying the requirement of free amino acids. Degradation of these peptides is required to establish a pool of amino acids which is presumably utilised for the synthesis of starvation specific proteins (Reeve *et al.*, 1984; Matin, 1990). Reeve *et al.* (1984) also concluded that *de novo* protein synthesis in *E. coli* and *S. typhimurium* on the onset of starvation provides proteins necessary for prolonged survival of the cells under starvation stress. Horan *et al.* (1981) demonstrated that amino acids (obtained by the degradation of

internal proteins) are used as endogenous substrates. One other possible function of stress-specific proteins has been reported by Kusser and Ishiquro (1985) that some proteins are produced under starvation stress which prevent the destructive effect of autolytic enzymes.

There has been much work on protein pattern changes of *E. coli*, *S. typhimurium* and some marine bacteria under starvation stress. In *E. coli* at least 30 polypeptides, including cytoplasmic and outer membrane proteins, are induced under carbon starvation (Groat and Matin, 1986). In marine bacteria, such as *Vibrio* sp., some novel outer membrane and cytoplasmic proteins are produced in oligotrophic environments. The rate of proteolysis is 16 times higher during the early stage of starvation than during exponential growth (Nyström *et al.*, 1988). Starvation proteins can make up to 5% of the total proteins in starved cell (Schultz *et al.*, 1988). Some starvation proteins were induced transiently at the onset of starvation, whereas others showed a more persistent pattern with high level at different times (Groat and Matin, 1986).

E. coli produce some common stress proteins under carbon, nitrogen or phosphorus starvation which have been designated as PEX proteins (Schultz *et al.*, 1988). It has also been shown in *S. typhimurium* that six common proteins were synthesised under nicotinate, phosphate and ammonium starvation (Spector *et al.*, 1986). *E. coli* (Groat and Matin, 1986), *S. typhimurium* (Spector *et al.*, 1986) *Bacillus subtilis* (Hecker *et al.*, 1989) and *Vibrio* sp. (Nyström *et al.*, 1990) have similar genes producing similar proteins classified as general stress proteins. Under carbon, phosphate and nitrogen starvation 6 proteins are common to nitrogen and phosphorus, and 3 are common carbon and nitrogen starvation. Nyström *et al.* (1990) showed that 14 polypeptides were produced as a result of starvation for carbon, nitrogen and phosphorus.

The accumulation of guanosine-3'-diphosphate-5'-diphosphate (ppGpp) in bacterial cells at the early stage of starvation stress is called "stringent control" (Nyström *et al.*, 1990). The importance of the stringent response in starvation

survival has been demonstrated by Mach *et al.* (1989) who showed that *rel A*⁻ mutants of *E.coli* lost viability significantly faster than their isogenic *rel A*⁺ counterparts during amino acid limitation and subsequent multiple starvation. Kjelleberg *et al.* (1987) reported that there was slow synthesis, reduced protein turnover and RNA accumulation in *Vibrio* sp. under starvation stress controlled by the stringent response.

Starvation is not the only factor affecting protein synthesis in bacterial cells. Proteins can also be induced or their synthesis increased by heat, ethanol, hydrogen peroxide, osmotic stress (Jenkins *et al.*, 1988), detergents (Adamowicz *et al.*, 1991) and temperature shifting (Jones *et al.*, 1987). It has been shown that a sub-lethal concentration of hydrogen peroxide led to the induction of 30 proteins in *E.coli* (Jenkins *et al.*, 1988). In addition Clark and Parker (1984) suggested that osmotic changes affect protein synthesis of *E.coli* and induce a group of proteins. Detergents, such as sodium dodecyl sulphate (SDS) resulted in an induction of some proteins in *E.coli*. Adamowicz *et al.* (1991) reported that the addition of 5% SDS to growing cultures led to the synthesis of at least 4 *de novo* proteins, the switch off of 13 proteins, the increased synthesis of 15 proteins and the repression of 15 others. At the same time, this stress led to be the induction of some stress specific proteins different to the usual stress proteins. Hecker and Völker (1990) noted that osmotic shock was an effective inducer of general stress proteins.

Temperature is also an effective inducer of stress proteins of bacteria. Araki *et al.* (1991) suggested that the rate of protein synthesis was enhanced by temperature upshift in *Vibrio* sp. ANT-300. They also showed that this strain of *Vibrio* had temperature-related characteristics which were different in 0°C-grown cells than in cells grown at 13°C. They speculated that the changes in temperature may affect the synthesis of only a small number of proteins. When temperature is shifted there is a transient change in the rate of synthesis of a number of proteins which are induced by the promotion of transcription which

requires an alternative subunit of RNA polymerase. This is known to be due to the production of the *rpoH* (*htrP*) protein and called the heat shock response (Neidhardt and Van Bogelen, 1981; Grossman *et al.*, 1984). Temperature shifting can also lead to the induction of more than a dozen proteins, none of which are heat shock proteins (Jones *et al.*, 1987; Goldstein *et al.*, 1990). Jones *et al.* (1987) found that an instant changes of temperature from 37°C to 10°C caused to growth to cease for several hours and resulted in the reduction of a remarkable amount of protein. The synthesis of about two dozen proteins accelerated dramatically after a shift from 28°C to 42°C.

The experiments were carried out here to investigate the changes in whole cell protein of *E. coli* under starvation and different stress conditions. The changes in protein patterns of *E. coli* are likely to be related to survival. Stress-specific proteins may play an important role for *E. coli* entering dormant state and remaining viable but non-culturable as a strategy for prolonged survival in lake water.

8.1.2 Changes in outer membrane proteins

It is generally believed that the outer membrane of Gram-negative bacteria is very important in terms of bacterial physiology in determining the interaction between microbes and their environments. The outer membrane components play a vital role in the adhesion of bacteria to surfaces. Also it is involved in the uptake of essential nutrients, the release of toxins, exchange of genetic information with other cells, the exclusion of toxic substances and resistance to attack by other cells. Major outer membrane proteins alter significantly as growth conditions change (Lambert, 1988).

The cell envelope of Gram-negative bacteria consists of three layers; the inner membrane, peptidoglycan and the outer membrane. In *E. coli* K-12, under laboratory conditions possesses some major outer membrane proteins, such as OmpF, OmpC and OmpA, and lipoproteins (Osborn and Wu, 1980). OmpF

(molecular weight approximately 37,200) and OmpC (36,000) are the major outer membrane proteins in *E.coli* K-12 (Lugtenberg and Van Alphen, 1983). The OmpA is also a major protein in heat-shock outer membranes and its molecular weight is approximately 35,000 D (Hiddennach and Henning, 1975). Porins are tightly but non-covalently associated with peptidoglycan (Lambert, 1988). Nikaido and Nakae (1979) suggested that porin proteins play an important role in membrane transport. Porins are water filled, passive diffusion channels that allow small hydrophilic molecules (up to 600 D) to cross the outer membrane. On the other hand, OmpA has an important role in *E.coli* in stabilising the outer membrane and retaining the rod shape. Because, OmpA-deficient mutant cells are extremely unstable, do not have a rod shape and the outer membrane is not attached to the peptidoglycan (Sonntag *et al.*, 1978). OmpA is also involved with lipopolysaccharide as a receptor in conjugation (Havekes and Hoeckstra, 1976). OmpA mutants also show reduced overall transport rates for amino acids and peptides transport (Nikaido and Vaara, 1985).

External factors such as phosphate deficiency (Korteland *et al.*, 1982), general starvation (Lutkenhaus, 1977), osmolarity and the types of nutrient sources (Lugtenberg *et al.*, 1976) can alter the activity of porin proteins in the natural environments. In *E.coli* and *S.typhimurium*, the synthesis of OmpF and OmpC porins are controlled by osmotic pressure as well as temperature (Lugtenberg *et al.*, 1976). In addition to osmolarity of the growth medium, nutritional composition can also affect the synthesis of porin protein (Lugtenberg *et al.*, 1976). Chai (1983) showed that changes in the cell envelope components may affect cellular physiology and cell survival of laboratory grown cultures. This alterations will also be particularly important in the natural environment. Nikaido and Vaara (1985) reported that under osmotic pressure, bacteria produce only OmpC porin. OmpF may be used by enteric bacteria in a low osmolarity such as fresh water in order to survive. They also suggested that

wider diameter of the OmpF pore would increase the capacity for assimilation of nutrients from very dilute environments by enhancing the permeability coefficient.

Furthermore, a number of porin proteins occur in some strains under certain growth conditions. For example *phoE* is synthesised under lack of phosphate and the rate of phosphate uptake was much higher in *phoE*-containing cells than in OmpF-containing cells (Korteland *et al.*, 1982). Protein K occurs in encapsulated strains of *E. coli* (Paakanen *et al.*, 1979). Another porin, LamB is involved in the uptake of maltose (Bavoil and Nikaido, 1981). The *tsx* porin protein of *E. coli* may have a function in nucleoside transport (Hantke, 1976) and can also function as a porin for amino acid transport (Henzenroeder and Reeves, 1981). Lastly OmpD is effective for the permeability of sugars and disaccharide (Osborn and Wu, 1986).

In these present experiments, OmpC, OmpF, OmpA and the other outer membrane proteins of *E. coli* have been examined under different nutrient conditions, temperature, starvation and other stress conditions in lake water in order to investigate a possible link between survival and outer membrane proteins.

8.2 Results

8.2.1 Changes in whole cell protein patterns of *Escherichia coli* under starvation conditions in lake water.

8.2.1.1 Protein pattern changes of *Escherichia coli* under starvation stress at different temperatures.

The observations reported in Chapter 3 showed that temperature and starvation led to *E.coli* cells becoming viable but non-culturable at 37°C in lake water. Consequently, the protein patterns of *E.coli* were compared at day 0 and after 30 days of starvation stress at 37°C. A number of proteins show marked differences in intensity between the two gels. They have been marked and numbered on the figure 8.1a and 8.1b. Compared to 0 day control, while the intensity of 9 protein spots in *E.coli* increased, the intensity of 9 protein spots was reduced. The rest of the proteins remained relatively unchanged by the effect of starvation at 37°C in filtered-autoclaved lake water. The intensity of some protein spots such as R5, R6, R9, R10, R13, R14, and R19 appeared to be reduced during starvation at 37°C. The intensity of others such as I3, I7, I12, I16, I17, and I20 was increased due to starvation stress. These protein patterns may have a significant function in the protection of *E.coli* cells from the detrimental impact of starvation in lake water. Those proteins which were reduced in intensity may have been degraded for the synthesis of other proteins, which appeared to increase in quantity in *E.coli*. Although some researchers have reported that *E.coli* produce *de novo* proteins in response to starvation, here it was mainly the intensity of the protein which was increased. This could have been due to deficiencies of silver staining of two-dimensional gels failing to reveal small changes.

Figure 8.1 Protein pattern changes of *Escherichia coli* under starvation stress

E. coli was grown overnight in nutrient broth, harvested and washed three times with sterile lake water. The cells were resuspended in filtered-autoclaved lake water. 500 ml sterile lake water microcosms was inoculated to give an initial viable count of approximately 10^8 cfu/ml. The flasks were incubated in the dark without shaking at 37°C. 100 ml cells were centrifuged, the pellet was resuspended and sonicated in Tris-EDTA buffer to extract the proteins.

Whole cell proteins of *E. coli* were determined by two dimensional gel electrophoresis according to the method of O'Farrell. Gels were silver stained and quantitative image analysis were carried out on the densitometer from the gels. The protein patterns of unstarved cells (Fig. 8.1a) were compared with cells starved for 30 days (Fig. 8.1b)

Fig.8.1a

Acidic

Basic

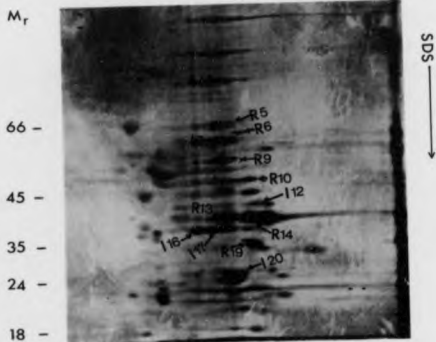
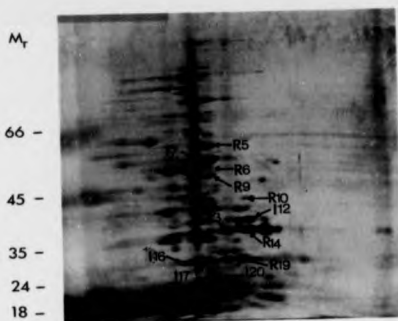


Fig.8.1b



8.2.1.2 The effect of phosphate starvation on protein synthesis of *Escherichia coli*

Inorganic phosphate limitation results in the synthesis of number of protein in *E.coli*. It has been shown that when *E.coli* was subjected to phosphate-limited conditions, the synthesis of 81 proteins was markedly changed (Wackett *et al.*, 1987; Filloux *et al.*, 1988). After growing *E.coli* in phosphate-limited minimal medium and transferring to lake water for 15 days some changes in protein patterns compared to control (normal grown) were seen. The changes are shown in Figure 8.2a and 8.2b. The protein spots that vary in intensity may be divided in three groups. Firstly those in which the intensity is increased strongly such as those spots numbered I10, I22, I23, I27, I33, I36, I38, I40, and I43; secondly, protein spots with reduced intensity such as those numbered R18, R21, R24, R25, R26, R30, R31, and R46; and lastly protein spots which remained unchanged. In general, the present experiments show that 22 proteins were induced by phosphate starvation, 16 proteins were reduced after 15 days of incubation in lake water at 15°C. Some protein spots such as A17, A47, A48, A49, A50 were only present in the pattern from cells allowed to develop from the phosphate-starved sample but were not present in the control sample from cells grown in minimal medium containing a sufficient amount of phosphate.

8.2.3 Changes in pattern of outer membrane protein synthesis in *Escherichia coli* under different conditions.

8.2.3.1 Outer membrane protein of *Escherichia coli* under starvation stress

In these experiments the changes in the outer membrane proteins, particularly of porins OmpF, OmpC and OmpA and an unidentified 45 kDa protein, of *E.coli* were examined over a 30 day period of starvation stress in lake water at 4°C, 15°C, 25°C and 37°C. It is generally accepted that outer membrane proteins, especially porins, establish an important link between

Figure 8.2 Protein pattern changes of phosphate-starved *Escherichia coli*

E. coli was grown overnight in minimal medium, harvested and washed three times with sterile lake water. The cells were resuspended in filtered-autoclaved lake water. 500 ml sterile lake water microcosms was inoculated to give an initial viable count of approximately 10^8 cfu/ml. The flasks were incubated in the dark without shaking at 37°C. 100 ml samples were centrifuged, the pellet was resuspended and sonicated in Tris-EDTA buffer to extract the proteins.

Whole cell proteins of *E. coli* were determined by two dimensional gel electrophoresis according to the method of O'Farrell. Gels were silver stained and quantitative image analysis were carried out on the densitometer from the gels. The protein patterns of minimal medium grown unstarved cells (Fig. 8.2a) were compared with the cells phosphate-starved (Fig. 8.3b).

Fig.8.2a

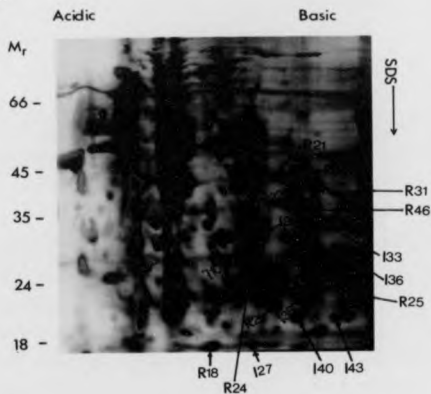
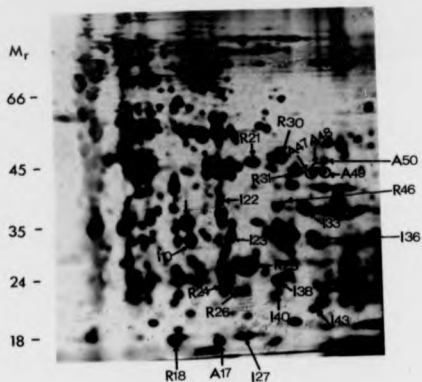


Fig.8.2b



Gram-negative cells and the environment. Changes in porin proteins could provide some selective advantage under poor nutritional conditions (Lutkenhaus, 1977).

Starvation stress produced changes in the outer membrane proteins of *E. coli* at different temperatures (Fig. 8.3a-e) and Table 8. 1a-d). The major protein in the outer membrane OmpF remained relatively constant in intensity (by visual inspection of the gels). This protein was therefore used as a marker with which to compare the relative amounts of the other major outer membrane proteins. The profiles of outer membrane proteins of cells starved for 38 days in lake water showed that the relative amount of a 45 kDa protein was increased by starvation at all temperatures (Fig. 8.3a-e). In this case, it was seen that the 45 ka protein increased in relatively terms by at least 10 times at 4°C and 37°C and at least doubled at 15°C and 25°C. At both 4°C and 37°C the relative amount of the 45 kDa protein increased with the length of the starvation period. This protein could play an important role in allowing nutrients to pass though the membrane or it could be a structural protein produced under starvation stress.

Another major outer membrane protein is the porin OmpC. Relative to OmpF this protein showed a variable pattern. Over the starvation period it probably shows a relative increase in abundance compared to OmpF. This increase is most obvious at 25°C and 37°C

The amount of OmpA present in the outer membrane declined. The band was barely visible on SDS polyacrylamide gels stained with Coomassie Blue especially at the higher temperatures after 30 or more days of starvation. Because the cell size reduction is greatest at the higher temperatures (Chapter 3) the decrease in OmpA could be related to the reduction in cell size.

The changes reported here are all dependent upon using a fixed standard. Visual observation of the gel showed that OmpF was always the most prominent band. However it was possible that the amount of OmpF present in the membrane of individual cells also changed. However as the same amount of

total protein was being loaded on each gel it was not possible to make any comment regarding the absolute abundance of OmpF during the starvation period. Differences in the staining or destaining regime between gels does not allow absolute comparison between gels run at different times.

8.2.3.2 Porin protein synthesis of *Escherichia coli* in lake water amended with nutrient sources.

As it was shown in Chapter 4 that *E. coli* survival was affected by the addition nutrients to lake water, the effects of some of these nutrient sources on the relative concentrations of outer membrane proteins were investigated. The results are shown in Figure 8.4 and Table 8.2. The patterns of outer membrane proteins are different. The addition of nutrients reduces the relative concentration of the 45 kDa and OmpA proteins compared to the control. The effect of nutrient addition on OmpA is most striking with the protein being reduced from a relative concentration of 0.5 compared to OmpF to between 0.22 (with glycine) and 0.06 (with glucose) relative concentration. Visually neither OmpF nor OmpC changes in concentration and the ratio of OmpF to OmpC remained relatively constant with only glucose addition having a significant effect on the ratio. Glucose addition reduced the ratio from close to unity to 0.67. These results suggest that the 45 kDa protein and OmpA are synthesised or increased in relative proportion in response to starvation and that the addition of nutrients which prolong survival of *E. coli* can alter the relative concentrations of these outer membrane proteins.

8.2.3.3 The effect of osmolarity and osmoprotectants on outer membrane proteins of *Escherichia coli*.

In this experiment the effect of osmolarity on the outer membrane proteins, particularly porin proteins, of *E. coli* was examined in filtered-autoclaved lake water at 37°C. Fig. 8.5 and Table 8.3 shows that starvation

Figure 8.3 Outer membrane protein pattern in *Escherichia coli* under starvation stress.

Outer membrane protein samples were prepared at different time periods during starvation in lake water, 50 to 100 μ g of protein was loaded on to 11% acrylamide with 3% stacking gel. Gels were stained with coomassie blue. Quantitative image analysis was carried out on a densitometer from the gels for major outer membrane porin proteins.

Figure 8.3a Outer membrane protein patterns of *Escherichia coli* cells after 60 days of starvation stress.

Track numbers;

1- control 2- 25°C 3- 37°C

Figure 8.3b-e Outer membrane protein patterns of *Escherichia coli* after starvation in lake water.

8.3b-4°C 8.3c-15°C 8.3d-25°C 8.3e-37°C

Track numbers;

1- Control (0. day)

2- 2 d 5- 17 d

3- 7 d 6- 25 d

4- 12 d 7- 31 d

8- 38 d

Fig. 8.3a

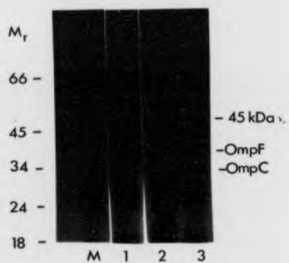


Fig. 8.3b



Fig.8.3c



Fig.8.3d



Fig.8.3e



Table 8.1a The effect of starvation stress on porin proteins of *Escherichia coli*
in lake water at 4°C

Days	OmpF Porin		45 kDa		OmpC Porin	
		Relative Amount		Relative Amount		Relative Amount
0	8,161	1.0	892.3	0.11	4,624	0.57
2	10,556	1.0	5,702.0	0.54	10,127	0.96
7	7,472	1.0	5,508.0	0.76	6,347	0.85
12	9,431	1.0	6,178.0	0.66	5,244	0.56
17	9,067	1.0	5,447.0	0.60	8,493	0.94
25	7,453	1.0	3,987.0	0.53	4,196	0.56
31	10,535	1.0	10,343.0	0.98	7,235	0.69
38	6,690	1.0	7,014.0	1.06	4,398	0.66

Table 8.1b The effect of starvation on porin proteins of *Escherichia coli*
in lake water at 15°C

Days	OmpF Porin		45 kDa		OmpC Porin	
		Relative Amount		Relative Amount		Relative Amount
0	2,212	1.0	355.1	0.16	777.8	0.35
2	3,749	1.0	972.4	0.26	2,484.0	0.66
7	6,633	1.0	1,920.0	0.29	5,412.0	0.82
12	5,704	1.0	1,636.0	0.29	4,688.0	0.82
17	4,788	1.0	3,643.0	0.76	2,659.0	0.56
25	4,045	1.0	1,318.0	0.33	2,600.0	0.64
31	4,165	1.0	2,067.0	0.50	1,588.0	0.38
38	4,271	1.0	1,269.0	0.30	2,142.0	0.50

Table 8.1c The effect of starvation on porin proteins of Escherichia coli in lake water at 25°C.

Days	OmpF Porin		45 kDa		OmpC Porin	
		Relative Amount		Relative Amount		Relative Amount
0	3,621.0	1.0	433.9	0.12	1,981.0	0.55
2	3,797.0	1.0	1,182.0	0.31	2,102.0	0.55
7	3,894.0	1.0	2,308.0	0.59	3,593.0	0.92
12	5,138.0	1.0	2,800.0	0.51	5,150.0	1.00
17	4,662.0	1.0	2,896.0	0.62	4,464.0	0.96
25	2,124.0	1.0	1,150.0	0.54	1,087.0	0.51
31	831.7	1.0	449.3	0.54	561.1	0.67
38	1,968.0	1.0	828.3	0.42	2,106.0	1.07

Table 8.1d The effect of starvation on porin proteins of Escherichia coli in lake water at 37°C.

Days	OmpF Porin		45 kDa		OmpC Porin	
		Relative Amount		Relative Amount		Relative Amount
0	4,579	1.0	329.0	0.07	4,142	0.90
2	3,739	1.0	1,041.0	0.28	1,798	0.48
7	3,887	1.0	1,284.0	0.33	2,852	0.73
12	2,134	1.0	628.7	0.29	1,950	0.91
17	3,351	1.0	500.9	0.15	1,789	0.53
25	1,500	1.0	956.7	0.64	1,413	0.94
31	4,108	1.0	1,670.0	0.41	3,020	0.74
38	3,662	1.0	2,972.0	0.81	3,837	1.05

Figure 8.4 SDS gel electrophoresis of outer membrane proteins of *Escherichia coli* cells starved in lake water amended with nutrient sources.

Outer membrane protein samples were prepared from *E.coli* cells incubated in lake water amended with nutrient sources after 5 days of incubation at 15°C. 50 to 100 µg of protein was loaded onto 11% acrylamide with 3% stacking gel. Gels were stained with coomassie. Quantitative image analysis was carried out on densitometer from the gels for major outer membrane porin proteins.

Track numbers:

1- D-alanine	2 mg/L	5- Lactose	2 mg-C/L
2- Glycine	2 mg-C/L	6- Glucose	2 mg-C/L
3- Glycerol	2 mg-C/L	7- Control	
4- Potassium nitrate	10 mg/L		

Fig.8.4

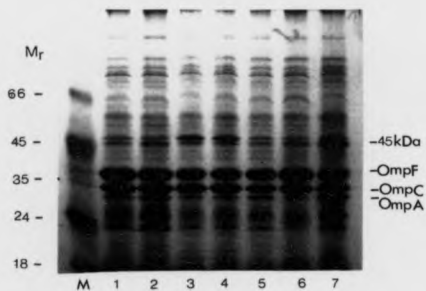


Table 8.2 Relative concentration of outer membrane proteins of *Escherichia coli* in lake water amended with nutrient sources at 15°C.

Nutrient Sources	OmpF Protein		OmpC Porin		OmpA
		Relative Amount		Relative Amount	Relative Amount
Control	1,432.0	1.0	1,568.0	1.08	719.3
D-Alanine	1,429.0	1.0	1,156.0	0.81	156.2
Glycine	1,410.0	1.0	1,317.0	0.93	308.1
Glyceral	910.9	1.0	1,052.0	1.16	101.1
Potassium nitrate	1,064.0	1.0	1,185.0	0.81	143.4
Lactose	1,113.0	1.0	896.6	0.67	165.3
Glucose	1,863.0	1.0	1,277.0	1.1	121.9

All readings were taken after 5 d starvation in lake water at 15°C

stress led to an increase in the relative amount of the 45 kDa protein (compared once again to OmpF as a standart). The relative amount of the 45 kDa protein present doubled in the first 72 hours of starvation. A large increase in the relative amount of this protein occurred in the presence of salt. The addition of 8% salt to the lake water resulted in this outer membrane protein becoming the dominant component of the outer membrane. The addition of osmoprotectants led to an increase in the relative amount of this protein over the control but it was reduced compared to the salt amended cells. The most effective osmoprotectants such as glycinebetaine and proline led to the smallest increase over the control. This suggested that this protein was produced in response to starvation and to osmolarity changes. There is no proof presented here that this protein was synthesised in response to osmotic shock. The same result would have been obtained if the amount of OmpF in the membrane had declined but the amount of this 45 kDa protein had remained constant. If this protein was to be synthesised in response to osmotic shock under these conditions it would once again suggest that the cells are viable and capable of protein synthesis even though the viable count is often undetectable after osmotic shock at 37°C.

The relative amount of OmpC present declined in the presence and absence of NaCl over the starvation period. In the presence of the best osmoprotectants, the relative amount of OmpC declined below that seen in the salt-stressed.

8.2.3.4 The effects chemical pretreatment on outer membrane proteins of *Escherichia coli*.

It has shown in Chapter 3 that pretreatment of *E.coli* in the growth medium with sodium dodecyl sulphate (SDS), hydrogen peroxide, sodium hypochlorite or ethanol caused some changes in the survival of *E.coli* in lake water. Whilst pretreatment with SDS, hydrogen peroxide or sodium

hypochlorite resulted in a rapid decline in the viable count after the transfer of *E. coli* to lake water microcosms, ethanol resulted in a rapid increase in the number of *E. coli*. The protein patterns of outer membrane proteins also showed changes compared to normal cells. As shown in Table 8.4 and Figure 8.6, the 45 kDa protein again increased in relative abundance upon starvation. The relative amount of the 45 kDa protein did not change significantly after pretreatment with hydrogen peroxide or sodium hypochlorite but pretreatment with ethanol did lead to a decline in its relative concentration. In the SDS-pretreated cells, however, the relative concentration of the 45 kDa protein was 16 times that of the control. This was due almost entirely to the loss of OmpF and OmpC from the outer membrane (Fig. 8.6).

The presence of OmpA in the outer membrane of *E. coli* was also unaffected by the pretreatment with any of the chemical compounds. OmpA disappeared under starvation stress, hence it is probably associated with the other stress conditions rather than only with starvation. Other differences in the outer membrane protein patterns were also seen on the gels. One notable protein band was specifically produced after hydrogen peroxide pretreatment of *E. coli* cells (Fig. 8.6). This protein was approximately 70 kDa in size and present only in the cells pretreated with hydrogen peroxide. This outer membrane protein could be involved in protecting cells from oxidative toxicity or their survival under oxidation stress.

It was previously shown that SDS pretreatment led to a drastic decline in the viable count of *E. coli* (Chapter 3). This could have been due to the dramatic alteration of the outer membrane especially the almost complete elimination of OmpC and OmpF from the outer membrane of *E. coli*. The effects of SDS-pretreatment on *E. coli* outer membrane protein patterns were also analysed with 2-D gel electrophoresis. In general 18 protein spots increased in density, 15 protein spots decreased with SDS pre-treatment (Fig. 8.7a and 8.7b). Some protein spots such as I2, I3, I5, I8, I15, I16, I18, I26, I27, I28 and I29 appeared much stronger in the pattern from the cells pre-treated with SDS. On the

Figure 8.5 The effect of osmolarity on the outer membrane protein of *E.coli* in lake water.

E.coli was grown overnight in nutrient broth at 30°C, harvested, washed with sterile distilled water and transferred to filtered-autoclaved lake water microcosms to give an initial viable count of approximately 10^7 cfu/ml. 8% NaCl was added to some flasks at the beginning of the experiment. Osmoprotectants or ammonium sulphate (0.1%) were added 24 h after inoculation. Flasks were incubated for 5 days in the dark without shaking at 37°C.

Outer membrane protein samples were prepared as described previously. 50 to 100 µg of protein was loaded on 11% acrylamide with 3% stacking gel. Gels were stained with coomassie blue. Quantitative image analysis was carried out on a densitometer from the gels for major outer membrane proteins.

Track number;

- 1- Control (0 day without NaCl)
- 2- 24 hours starved cells (without NaCl)
- 3- Control (with NaCl)
- 4- Ammonium sulphate added (0.1%) 3 days starved cells
- 5- Ammonium sulphate added 3 days starved cells (with NaCl)
- 6- Glycine betaine (17 mM)
- 7- Proline (4.3 mM)
- 8- Phosphorylcholine chloride (7.6 mM)
- 9- Acetyl-8-methylcholine chloride (10 mM)
- 10- Glutamate (2 mM)

Fig. 8.5

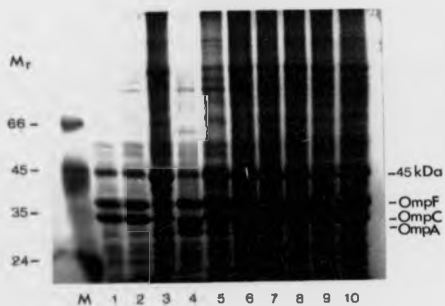


Table 8.3 The effect of osmolarity and osmoprotectants on outer membrane proteins of *Escherichia coli* in lake water

Samples	OmpF Porin		45 kDa		OmpC Porin	
		Relative Amount		Relative Amount		Relative Amount
Control	999.2	1.0	243.8	0.24	1,492.0	1.49
24 h starved cells	1,223.0	1.0	444.8	0.36	1,830.0	1.50
Control (+ NaCl)	1,204.0	1.0	3,197.0	0.42	997.8	0.74
72 h starved	1,789.0	1.0	755.7	2.66	1,317.0	0.83
72 h starved (+ NaCl)	5,704.0	1.0	2,542.0	0.45	4,110.0	0.72
Glycinebetaine	6,111.0	1.0	3,568.0	0.53	4,014.0	0.66
Proline	6,675.0	1.0	3,172.0	0.48	4,612.0	0.69
Phos.choline chloride	5,600.0	1.0	4,500.0	0.80	4,649.0	0.83
Acet.choline chloride	4,244.0	1.0	2,923.0	0.69	2,962.0	0.69
Glutamate	6,878.0	1.0	3,343.0	0.49	4,524.0	0.49

contrary, some protein spots such as those numbered R9, R11, R19, R24, R31, R32 and R33 came out with less intensity compared to the control gel sample. Rest of the proteins did not show any significant changes with SDS pretreatment. Protein spots such as I18, I26, I27 may have an important function in cells under SDS stress. Furthermore some proteins disappeared such as those D20, D32, D33, D34, D37, D38 and D42 and some of the others, such as those numbered A39, A40, A41 and A43 only appeared in SDS pre-treated *E. coli* cells.

Figure 8.6 The effect of chemical pretreatment on outer membrane proteins of *Escherichia coli*

E. coli was grown in nutrient broth with and without the addition 18 mM hydrogen peroxide, 0.5% sodium dodecyl sulphate or 1% ethanol overnight at 30°C, harvested, washed and resuspended in tris-EDTA buffer pH 8.5. The samples were sonicated and centrifuged and outer membrane protein samples were prepared as described. 50 to 100 µg outer membrane protein samples were loaded onto 11% acrylamide with 3% stacking gel. Gels were stained with coomassie blue. Quantitive image analysis was carried out on a densitometer from the gells for major outer membrane proteins.

Fig.8.6

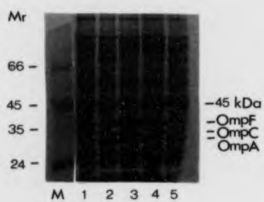


Table 8.4 The effect of pretreatment with hydrogen peroxide, SDS, sodium

hypochlorite and ethanol on outer membrane proteins of *Escherichia coli*.

Pretreatment	OmpF Porin		45 kDa		OmpC Porin	
		Relative Amount		Relative Amount		Relative Amount
Control	6,557.0	1.0	7,254	1.11	4,165.0	0.64
Hydrogen peroxide (18 mM)	6,866.0	1.0	6,692	0.97	3,640.0	0.53
Sodium hypochlorite(0.29 M)	12,681.0	1.0	9,847	0.78	10,443.0	0.82
Ethanol 1%	18,904.0	1.0	10,998	0.58	14,154.0	0.75
SDS (0.5%)	426.9	1.0	7,212	16.89	27.1	0.06

Figure 8.7 Changes in outer membrane protein pattern of *Escherichia coli* pretreated with sodium dodecyl sulphate.

E.coli was grown at 30°C overnight in nutrient broth amended with and without 0.5% (w/v) SDS, added 5 h after inoculation. Cells were harvested, washed three times with sterile lake water. Cells were centrifuged, sonicated in Tris-EDTA buffer, pH 8.5. Outer membrane protein samples were prepared as described. Protein samples were loaded onto tube gel for the first dimension (isoelectric focusing). The gels were removed and treated with SDS prior to running. Protein spots were visualised using silver staining. Quantitative image analysis was carried out on in a densitometer.

Figure 8.7a Outer membrane protein patterns of normal grown *Escherichia coli* cells (control)

Figure 8.7b Outer membrane protein patterns of *Escherichia coli* cells pretreated with SDS

Fig. 8.7a

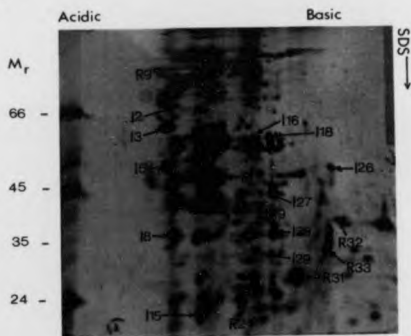
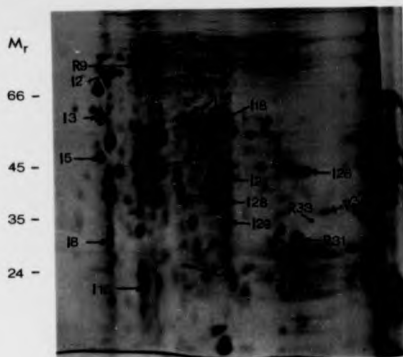


Fig. 8.7b



8.3 Discussion

The experiments were carried out here showed that there was an alteration in the outer membrane protein patterns of *E.coli* under starvation stress in lake water at 37°C. As it was reported in previous chapters that this temperature appeared quite critical for *E.coli* survival and led to a rapid disappearance of *E.coli* in lake water due to cells becoming viable but non-culturable. These changes in the protein patterns of *E.coli* are consistent with the findings reported by Reeve *et al.* (1984), Groat and Matin (1986), Schultz *et al.* (1988) and Matin (1990) who showed that some *de novo* proteins are produced in *E.coli* or some proteins are switched on or off under nutrient deprivation conditions. Quantitative image analysis revealed that there were 9 induced proteins, 9 repressed proteins in *E.coli* because of starvation stress at 37°C. It was considered unlikely that *de novo* protein synthesis occurred, because *E.coli* inoculated into the lake water starvation medium were in stationary phase. The changes protein patterns of *E.coli* in lake water under starvation stress could be explained if there were some proteins in cells which could be degraded in response to specific environmental changes. It is also possible *de novo* synthesis occur with some proteins being degraded during starvation to provide free amino acids in the cell which can be used to synthesise the necessary starvation proteins (Reeve *et al.*, 1984). This conclusion is also supported by Horan *et al.* (1981) who suggested that amino acids obtained from proteins under starvation stress could be used as endogenous substrates.

The effect of nutrient amendments on the protein patterns of starved *E.coli* was also examined. There has been previous work on the effect of phosphate starvation on bacterial proteins (Wackett *et al.*, 1987; Schultz *et al.*, 1988; Filloux *et al.*, 1988). When *E.coli* become phosphate-limited the synthesis of 81 proteins was markedly enhanced and half of these proteins were new

proteins (Wackett, 1987). Garen and Levinthal (1960) showed that under phosphate limitation up to 6% of cell protein in *E. coli* were specific to this limitation. Using isoelectric focusing 2-D gel electrophoresis results also demonstrated that 7 new protein spots appeared to be strongly enhanced in phosphate-starved cells. In addition, the concentration of 16 proteins was reduced and of 22 was increased according to quantitative image analysis compared with controls grown under normal conditions. These results suggested that phosphate starvation caused variations in protein synthesis, which might be related to conserving energy consumption or phosphate scavenging under starvation stress. The specificity of the new protein patterns were determined. These could be general stress proteins or specific to phosphate-limited cells. These protein patterns, however, might reflect the true protein patterns of *E. coli* from aquatic environment as most of these are usually phosphate limited.

Changes to outer membrane proteins, particularly porin proteins, of *E. coli* were examined under starvation and stress conditions in lake water microcosms at different temperatures. The outer membrane is the first important barrier between Gram-negative bacteria and their surrounding environment. The composition of the membrane can be altered quantitatively and qualitatively when cells are exposed to different nutritional conditions in the natural aquatic environments. Cellular physiology and bacterial survival can be affected by changes in the cell envelope which could be particularly important in the natural aquatic environment (Chai, 1983).

Studies have been conducted on porin proteins in different bacterial species and have shown that there were some changes in porin proteins in terms of the cells permeability to different nutrient sources, and their resistance to toxic chemicals and inhibitors (Nikaido and Nakae, 1979). Others have also shown that different stress condition, such as osmolarity (Hasegawa *et al.*, 1976; Nikaido and Vaara, 1985), starvation stress (Korteland *et al.*, 1982; Nikaido and Vaara, 1985) and temperature (Lugtenberg *et al.*, 1976) can affect

porins. Van Alphen and Lugtenberg (1977) showed that the relative amount of porin proteins present in the membrane is dependent on the growth temperature, the growth phase and medium composition. Our results demonstrated that there were a significant changes in outer membrane proteins, especially porin proteins, in *E. coli* cells under starvation conditions at different temperature. The most notable change was the induction of unidentified approximately 45 kDa outer membrane presumed to be a porin protein which only appeared under starvation stress. Moreover this porin protein was induced in proportion to the length of starvation period in lake water at all the incubation temperatures. The 45 kDa protein was also present in *E. coli* grown in minimal medium. There are reports in the literature that some outer membrane porin proteins are only synthesised under specific conditions. For example, *phoE* is induced under phosphate starvation (Korteland *et al.*, 1982), the K porin is synthesised by encapsulated *E. coli* (Paakanen *et al.*, 1979) and LamB is synthesised for maltose uptake (Benz *et al.*, 1979). In the case of the synthesis of the 45 kDa protein in *E. coli*, one can argue that a factor, probably starvation stress in lake water, results in the induction of this protein.

The amount of the OmpF porin in outer membrane of *E. coli* under starvation stress appeared to show a gradual decline during the course of experiments in lake water. The highest decline occurred during the first 24 h of incubation and then little further fluctuation was observed. The lowest reduction occurred at 37°C compared to other incubation temperatures. It has been reported that OmpF and OmpC are effective in conferring permeability to sugars and disaccharides (Osborn and Wu, 1980). It is possible that the wider diameter OmpF porin (Nikaido and Vaara, 1985) can be an advantage to *E. coli* for the assimilation of nutrients from dilute environment such as lake water, but under starvation stress, reduction of the cell size might affect the need for the large pore size of the OmpF.

The amount of OmpC porin protein in outer membranes of *E. coli* was increased in the first few days of starvation stress in lake water then started to decline at 4°C and 15°C while remaining relatively constant at 37°C. OmpC porin has the same function as OmpF in *E. coli* which transport nutrients through the membrane (Osborn and Wu, 1980). However there was not significant changes in the amount of OmpF porin protein in *E. coli* under starvation stress.

OmpA protein, which is a major constituent of the *E. coli* outer membrane disappeared after 24 h of incubation under starvation stress in lake water at different incubation temperatures. This phenomenon did not show temperature dependency. It has been reported that OmpA is similar to porins and its function is to stabilise outer membranes and maintain the rod shape of *E. coli*. The envelope of mutants of *E. coli* lacking this protein is extremely unstable and the *E. coli* cells do not have usual rod shape and the outer membrane unattached to peptidoglycan (Sonntag *et al.*, 1978). Here it is possible that the reason for the disappearance of OmpA in *E. coli* could be explained by the reduction in cell size which occur. OmpA has to be lost of the usual rod shape is change. Moreover, the disappearance of OmpA protein occurred gradually as does cell size reduction. There is a relationship between the length of the starvation period and the nutritional status of lake water must affect the survival of *E. coli*. Therefore outer membrane proteins were also examined after the individual addition of nutrient sources to the lake water. Nutrient sources, such as glucose, glycine, glycerol, lactose, potassium nitrate and D-alanine, led to a relative increase in OmpF in contrast to starvation conditions. The synthesis of OmpC was also influenced by the addition of nutrient sources to the lake water at 15°C. OmpA again disappeared, even after the addition of nutrient sources to lake water microcosms. However, the addition of glycine had little influence on OmpA compared to other additions.

The concentrations of porin proteins in the outer membrane of *E. coli* were also analysed under conditions of osmotic stress in lake water at 30°C. It

has been previously reported that the synthesis of the porins, OmpF, OmpC, was regulated by a combination of the osmolarity of the medium and the incubation temperature (Hasegawa *et al.*, 1976). Nikaido and Vaara (1985) reported that the addition of 10% sucrose resulted in the repression of OmpF at 42°C. Although 0.9% NaCl is equivalent to 10% sucrose in terms of osmotic pressure, the relative amount of OmpC present in the outer membrane declined in the presence of salt over the starvation period. The addition of osmoprotectants further decreased the relative concentration of OmpC compared to OmpF in the presence of salt. This could have been due to either the selective inhibition of OmpC synthesis, loss of OmpC from the membrane by solubilization or due to an increase in the concentration of OmpF present with no concomitant increase in OmpC.

The relative amount of 45 kDa protein present in the outer membrane was also dependent upon the degree of osmotic shock which the cells suffered. Although the relative amount of the 45 kDa protein increased upon starvation, the amount increased further in salt-stressed cells. This again could have been due to a genuine increase in the 45 kDa protein relative to OmpF or due to a decrease in the OmpF protein relative to 45 kDa protein. After a period of starvation in salt-amended water the 45 kDa protein became the dominant protein in the outer membrane. The presence of osmoprotectants, such as glycinebetaine and proline, decreased the amount of this protein relative to those cells subjected to salt stress in the absence of osmoprotectants. It is suggested that this outer membrane protein is produced in response to shock as it is present in increased amounts in the outer membrane in cells subjected to osmotic shock and to starvation stress. Relieving part of the stress caused by osmotic shock by the use of osmoprotectants reduces the relative amounts of this protein in the outer membrane closer to the level seen in cells subjected only to starvation stress. If this 45 kDa protein was genuinely synthesised in osmotically-shocked

cells, it implies that these cells are capable of protein synthesis even though they are undetectable by plate counts as shown in Chapter 3.

Pretreatment of *E. coli* with hydrogen peroxide, sodium hypochlorite, ethanol and SDS before transfer to the lake water led to changes in the outer membrane proteins of *E. coli*. Hydrogen peroxide resulted in an induction of outer membrane protein (approximately 70 kDa in size) which was not present in the control sample. This outer membrane protein was also not observed under other stress conditions. This new protein could have a function to protect *E. coli* cells from oxidation stress and could be involved in maintaining *E. coli* survival in lake water. It has been reviewed by Farr and Kogoma (1991) and Jenkins *et al.* (1988) that 2-D protein analysis of *E. coli* cells treated with hydrogen peroxide revealed that some proteins were synthesised at an elevated rate after treatment. Ethanol pretreatment did not lead to a dramatic changes in outer membrane protein by SDS-PAGE technique, but the relative concentration of the 45 kDa protein was decreased or OmpF and possibly OmpC increased. This could be an explanation for the viable count of *E. coli* cells rapidly increasing in lake water after pretreatment with ethanol (chapter 3). Ethanol pretreatment could have resulted in an increase in the nutrient scavenging capability of *E. coli*.

The most significant alterations in protein bands were observed in *E. coli* pretreated with SDS and transferred to lake water microcosms. It has been reported that enteric Gram-negative bacteria which live in the intestinal tract of animals are protected from the detergent effect of bile salts and the degradation by digestive enzymes by their outer membrane barrier (Nikaido and Nakae, 1979). The present experiments showed that SDS pretreatment led to a dramatic decline in the viable count of *E. coli*. OmpF and OmpC proteins disappeared from the outer membrane of *E. coli*. The results suggested that OmpF and OmpC could play an important role in terms of the survival of *E. coli* in the presence of detergents. The loss of these porins may lead to a difficulty for the cells in transferring necessary molecules through the membrane efficiently. As

consequence *E.coli* lose viability in lake water after pretreatment. Outer membrane protein patterns of *E.coli* were also examined by 2-D gel electrophoresis and showed that 8 new proteins were present, 4 proteins were absent, 18 proteins were increased in intensity and 15 proteins were decreased according to their measurement by image analysis. These results confirmed previous reports SDS caused protein pattern changes in *E.coli* cells (Adamowicz *et al.*, 1991).

In brief, it must be noted that the OmpF, OmpC, OmpA and 45 kDa protein and OmpA in *E.coli* can be altered quantitatively and qualitatively under adverse environmental conditions in lake water. These changes in major porin proteins tend to be made as alternatives to each other to maintain cell survival. When some of porins decline, the others are increased. The most important results was that the 45 kDa protein increased in intensity with an increasing the length of starvation period to compensate for the decline in OmpC and OmpF proteins in lake water.

Chapter IX. General discussion

Several biotic and abiotic factors have been considered as the main parameters affecting the survival of faecal bacteria in aquatic environments. The survival of a microorganism in an environment in which it is not indigenous is dependent upon the organism's ability to tolerate a set of biological and physicochemical factors those in its native habitat. In general those factors which have been considered include solar radiation (Chamberlain and Mitchell, 1978; Barcina *et al.*, 1986), temperature (McFeters and Stuart, 1972; Flint, 1987), inadequate concentrations of nutrients (Morita, 1985; Lim and Flint, 1989; Henis *et al.*, 1989), competition for nutrients (Flint, 1987) and protozoa predation (Gurijala and Alexander, 1990).

The addition of nutrient sources to untreated and filtered-autoclaved lake water caused some alterations on the survival of *E.coli*. The addition of carbohydrates allowed *E.coli* to grow in filtered-autoclaved lake water suggesting that in the absence of the natural microflora there was sufficient nitrogen and phosphorus sources to allow growth to occur if a suitable carbon source was added to the water. The addition of some carbohydrates, for instance sucrose and lactose to untreated lake water and mannose, galactose and fructose to filtered-autoclaved lake water led to a more rapid decline in bacterial numbers once decay commenced. This could be due to the high concentrations of carbohydrates used leading to unbalanced cell growth and death due to the absence of sufficient nitrogen and phosphorus sources to meet to the demand for these nutrients. The addition of carboxylic acids such as acetate, propionate and pyruvate as carbon sources clearly enhanced the survival of *E.coli* in untreated lake water at 15°C. This suggests that these metabolic intermediates could be taken up or used more efficiently than sugars by these stressed bacteria (Brown, 1972). The addition of amino acids as carbon sources had little effect on the survival of *E.coli* in the presence of the natural microflora of the lake. In filtered-autoclaved lake water, some amino acids could act as growth substrate

leading to growth of the bacteria in lake water. The most profound effect of nutrient additions on survival of *E.coli* occurred with ammonium sulphate and casein additions. The additions of increasing concentrations of these substances led to a concentration dependent increase in the survival times of *E.coli* in untreated lake water. Similar results have been reported by Lim and Flint (1989). The reason for the increased survival of *E.coli* in the presence of these nitrogen sources remains unclear. *E.coli* does not have a means of storing nitrogen and hence it might be expected that cells would require a nitrogen source when they are stressed under starvation conditions. It is likely that *E.coli* has a very low affinity uptake system because the concentrations of ammonium ions which have most effect are very high suggesting that uptake is by passive diffusion. The natural ammonium ion concentrations of water, *E.coli* is unlikely to be able to compete with the indigenous microflora for the natural low concentrations of ammonium ion present in the water. The indigenous microflora will have adapted to survive in the presence of low ammonium ion concentration in lake water. The fact that ammonium ion is not acting as a growth substrate can be seen by the lack of growth when it is added to filtered-autoclaved lake water.

E.coli is one of the important indicator organisms for faecal pollution. The presence of *E.coli* in water implies that other pathogens are also present in aquatic environments. Here synthetic sewage additions to lake water resulted in an increase in the survival of *E.coli* in lake water at 15°C and 30°C. This was consistent with the findings of Flint (1987) and Lim and Flint (1989). *E.coli* was able to grow if they were discharged from a sewage works into an aquatic environment. Growth only ceases when nutrients becoming limiting and *E.coli* can no longer compete with the indigenous microflora. Lim and Flint (1989) also suggested that temperature was an important parameter for determining the survival of bacteria in sewage amended water. Here *E.coli* could grow in lake water amended with sewage at 15°C and 30°C but not at 4°C. *E.coli* would be

expected to have very low metabolic activity at this low temperature and hence to be unable to grow. It is also possible that protozoa play a role in removing bacteria from the samples. Again they would have a higher metabolic activity at the higher temperatures but it has also been shown that only slow growing bacteria (i.e. *E.coli* at 4°C) are eliminated by protozoa whereas those which have a faster growth rate can survive (Sinclair and Alexander, 1989).

The results of the survival experiments in lake water microcosms suggest that the indigenous community in lake water play a role in the reduction in bacterial numbers. Many researchers have reported that protozoa predation was the main factor causing death of *E.coli* in aquatic systems (McCambridge and Meekin, 1979, 1981; Barcina *et al.*, 1986; Rhodes and Kator, 1989; Garcia-Lara *et al.*, 1991). Using metabolic inhibitors to inhibit protozoan activity had no effect on the survival of *E.coli* in our microcosms hence we eliminated protozoa as an important factor in survival under these conditions and in these water samples. However filtration of the water through Millipore filters to remove all bacterial competitors did have an effect on the survival of *E.coli*. However survival was still lower than in sterile water samples suggesting that bacteriophage play a minor role in bacterial decay. The addition of lytic bacteriophage to the water samples did reduce survival times but only using high titres.

E.coli survival was also examined under the effect of different stress conditions. There were some physiological and morphological alterations in *E.coli* cells when they were exposed to starvation conditions in lake water. However the survival experiments demonstrated that *E.coli* survival was affected not only by starvation stress but also by the incubation temperature. *E.coli* survived for the longest period of time at 4°C and for the shortest period of time at 37°C according to plate counts (i.e. viable counts). There was little difference in any survival times below 25°C (similar to the results obtained by Flint (1987) for river water). It has been suggested here that 37°C is a critical

temperature for the survival of *E. coli* in lake water as at this temperature *E. coli* was more likely to enter a viable but non-culturable state. Although the plate counts declined rapidly at 37°C the ability of the cells to respire was lost less rapidly and respiring cells could still be detected in the water samples after 96 days long after the viable count had become undetectable. Morphological changes to the cell size and shape were also observed more rapidly at 37°C using epifluorescence microscopy, transmission electron microscopy and Coulter counting. Only small changes occurred to the cells at the lower temperatures but the viable count also did not decrease. The entry into the viable but non-culturable state has to be a result of the reduction in cell size. The temperature dependent entry into the viable non-culturable state has also been shown for *V. vulnificus* (Wolf and Oliver, 1992). Our results suggest that *E. coli* cells undergo some changes common to all bacteria in order to protect themselves from the effects of starvation stress.

The effects of other stresses on the survival of *E. coli* were also examined. A sudden change from fresh water to salty water leads to dehydration of the bacterial cells and damage to the cell membrane (Sleyter and Messner, 1983). Lim (1988) noted that in 0.8 M salt-amended lake water *E. coli* survival was decreased and that this decrease was due to a more rapid decline in viability than due to a decreased lag phase before the reduction in cell numbers began. Here we have shown that this severe loss of viability could be prevented and to some extent reversed with the addition of osmoprotectants such as glycinebetaine and proline to the lake water. Again this suggests that the results of stress is not death of the cells but rather the loss of the ability to grow on agar plates. Direct counts and respiring cell counts again suggest that the cells retain their viability but are non-culturable. Other stresses, such as chlorine stress, hydrogen peroxide, sodium dodecyl sulphate and ethanol stress yielded essentially similar results. In all cases the cells lost the ability to grow on agar plates but could recover if the stressor was removed and respiring cell counts

still showed that a large proportion of the cell numbers could respire. It is generally agreed that chlorine stress is the most important in terms of the detection of *E. coli* in water samples (LeChevallier *et al.*, 1985). If cells are merely damaged rather than killed by chlorination then the use of *E. coli* as an indicator of the potability of drinking water becomes questionable.

Electron transport system (ETS) activity was examined in *E. coli* with and without the addition of nutrient sources to filtered autoclaved lake water. Several researchers have shown that the respiration rate of bacteria decline under starvation stress in aquatic environments in response to starvation (Boylan and Ensign, 1970; Novitsky and Morita, 1977; Smigielsky *et al.*, 1989). ETS activity was highest in cells incubated in sterile lake water at 37°C than at 4°C. However up to 65% of ETS activity was lost at 37°C within 24 hours of starvation compared to at 4°C where a comparable loss of activity was observed after 6 days incubation. Although the viability of the cells at 4°C and 15°C did not decline their respiration rate did decline. At 37°C the viable count ultimately became undetectable but there was still measurable respiration activity. Again these results imply that *E. coli* becomes non-culturable rather than dead under the effects of starvation stress. The addition of nutrients to the water led to higher respiration activity even though there was not always a change in viable count. This suggests that the cells could utilise the nutrients to support essential cell functions but not generate enough new biomass for cell growth.

The metabolic activity of *E. coli* was also measured by determining the activity of the enzymes succinate dehydrogenase and NADH dehydrogenase in cells under starvation stress at different incubation temperatures in sterile lake water. The activity of these enzymes was lost more quickly at 37°C than at other temperatures. This parallels the loss in ETS activity, the loss in numbers by plate count determinations and the reduction in cell size seen in other experiments. The activity of these enzymes remained virtually unchanged at 4°C, the temperature at which there was also little loss in ETS activity or in

viability as determined by plate counts, and little reduction in cell size. This again supports our belief that *E. coli* enters a viable but non-culturable state more readily at 37°C than at 4°C. The addition of carbon sources resulted in an increase in the activity of these enzymes and of ETS at 37°C. However the addition of nitrogen sources had little effect on enzymes activity although it was these additions which prolonged *E. coli* survival. It is probable that the addition of nitrogen sources particularly ammonium ions prevents the entry of *E. coli* into the viable but non-culturable state and maintains a high level of viability of the cells. There was a strong correlation between viability as determined by plate count, dehydrogenase activity and ETS activity and cell size reduction of *E. coli* held at 37°C under starvation stress.

Alkaline phosphatase is an important enzyme for the survival of bacteria which have become phosphate limited. It is usually only synthesised in the absence of inorganic phosphate in the growth medium. The enzyme is therefore produced in response to a form a starvation stress i.e. the unavailability of a specific growth nutrient. Here alkaline phosphatase activity was measured under the effects of long-term starvation stress and the effect of other stresses such as heat shock, osmolarity changes and oxidation stress. Alkaline phosphatase activity increased as the starvation period increased at of temperatures between 4°C and 37°C. Similar results had been reported for *E. coli* starved in sterile seawater (Munro *et al.*, 1989; Gauthier *et al.*, 1990). Here the increase in activity occurred with no increase in cell numbers and at temperatures such as 4°C at which protein synthesis in *E. coli* would be expected not to occur. Activity also increased at 37°C despite the reduction in viable count to almost undetectable levels. This increase could be due to *de novo* enzyme synthesis but as there was also an increase at 4°C. This seems unlikely as there is no evidence that *E. coli* can carry out protein synthesis at this temperatures. The increase could be due to starvation induced changes in the outer membrane of *E. coli* allowing the substrate easier access to the enzyme which is located in the

bacterial periplasm. The enzyme is known to be very stable and it is unlikely that activity is lost even if the cells actually die rather than simply become non-culturable. The addition of nutrient sources to lake water also lead to an increase in suggesting that the cells were still able to carry out protein synthesis under starvation conditions with the addition of a single carbon or amino acid source to the sterile lake water. The addition of ammonium sulphate and ammonium nitrate did not lead to an increase in enzymes activity although again the disappearance of the viable count was halted. Again this suggests that the effect of ammonium sulphate is not on cell metabolism rather it seems to stabilise the cells and prevent loss of viability.

When *E.coli* was exposed to osmotic shock again alkaline phosphatase activity increased. The activity was proportional to the salt concentration added to the sterile lake water. There was no increase in activity in cells grown in high phosphate only in cells which were already derepressed for alkaline phosphatase synthesis. This suggests that osmotic shock does not lead to derepression of enzyme synthesis but simply increase the synthesis of an already derepressed enzyme. Alternatively the increase in activity could again be due to osmotically-induced changes in the outer membrane allowing easier access to the periplasm. The addition of osmoprotectants, however, prevented the increase in alkaline phosphatase activity. These osmoprotectants function inside the cell rather than at the membrane level. Hence the effect seen here is rather one associated with enzyme synthesis or formation of the active enzyme from the inactive monomers rather than with changes in membrane permeability. Heat shock did not lead to increase in alkaline phosphatase activity but as the plate count became undetectable there was no loss in activity This again shows the stability of this particular enzyme. Pretreatment *E.coli* with ethanol or hydrogen peroxide as other stressors also increased the alkaline phosphatase activity of the cells. It is possible that alkaline phosphatase could be part of the global response to shock

network rather than simply being synthesised when phosphate is limiting i.e. a limited part of the starvation stress response.

A method based on the hydrolysis of the substrate o-nitrophenyl- β -D-galactoside (ONPG) by the β -galactosidase enzyme of faecal coliforms can be used as a very rapid and accurate method for the determination of these bacteria in aquatic environments (Warren *et al.*, 1978; Edberg *et al.*, 1990, Manafi *et al.*, 1991). However it is known that bacterial enzymes are strongly affected by their nutritional, chemical and physiological environment. Hence it is possible that stressed bacteria may not produce β -galactosidase under the test conditions and hence remain undetectable in the environment. Therefore the activity of β -galactosidase was studied under different temperatures in sterile lake water. The β -galactosidase activity of *E. coli* was affected by starvation stress with activity declining as plate counts declined. Activity was detectable for up to 60 days in cells starved at 4°C and 15°C but was undetectable after 13 days in cells starved at 25°C or 37°C. Some nutrient sources particularly amino acid additions and ammonium ions led to an increase in β -galactosidase activity and to a longer period before they became undetectable. This was followed by a longer period of viability as determined by the plate count. It would appear from these results that as the bacteria become more stressed and enter the viable non-culturable phase so they lose β -galactosidase activity. This suggests that the enzyme is not as stable as alkaline phosphatase and that it is probably degraded to provide nutrients to the starving cells. The addition of nutrients prolongs the stability of the enzyme by satisfying part of the cells nutrient requirement hence β -galactosidase is not broken down.

In the final set of experiments the changes to the total and membrane protein patterns of *E. coli* were followed during starvation and other stresses using 2-D gel electrophoresis and the usual SDS-PAGE method. Starvation stress resulted in the induction of some proteins and the repression of others. It is possible that the protein spots which disappeared were degraded to provide

amino acids for the synthesis of new proteins necessary to maintain cellular integrity under starvation conditions. Similar results have been reported by Reeve *et al.* (1984). Using quantitative image analysis it could be seen that under conditions of phosphate starvation, 7 new proteins appeared and 16 were reduced in intensity or disappeared completely. These newly synthesised proteins would be part of the well established response of the cell to phosphate starvation.

Further studies on the proteins particularly porins of the outer membrane of *E. coli* showed that these also altered in response to starvation and other stresses. The most noticeable change was the induction of an unidentified 45 kDa protein which appear in the outer membrane only under starvation conditions. The amount of OmpA, OmpC and OmpF in the outer membrane were compared using quantitative image analysis. OmpC appeared to increase initially in response to starvation stress but then began to decline. OmpF was more constant throughout the starvation period. Unfortunately there is no quick method of determining the absolute amount of any one porin in the outer membrane rather changes were monitored by following the changes in the relative proportion of each porin present. OmpA almost disappeared from the outer membrane under the effects of starvation stress. This outer membrane protein has a role in maintaining cell shape (Sonntag *et al.*, 1978). It is perhaps no surprise that as the cell begins to undergo miniaturisation as it enters the viable non-culturable state then this protein is lost from the outer membrane.

Pretreatment of *E. coli* with hydrogen peroxide, SDS and ethanol as other stressing agents resulted in changes in the outer membrane which again could be correlated to the survival of the cell under stress conditions. The use of oxidative stress resulted in the induction of an outer membrane protein of approximately 70 kDa molecular mass which was not present in the control samples. This protein was not observed under other stress conditions. The most significant changes occurred with SDS which removed almost all the major

outer membrane proteins. This was accompanied by a rapid reduction in the viable count. However other new proteins were synthesised and could be responsible for maintaining cellular integrity under the stress imposed by detergents such as SDS.

Overall this project has been an investigation into some of the many physiological and morphological changes which must occur in *E.coli* cells subjected to starvation and other stresses. The other stresses were examined to provide a comparative survey of the effects of stress on bacterial cells. Some stresses, for instance heat and oxidative shock are well documented. We believe that we have shown here that the entry of *E.coli* into the viable but non-culturable state is temperature dependent. It occurs more readily at temperatures at which is physiologically active rather than at 4°C, for instance, at which temperature *E.coli* is not capable of protein synthesis amongst other functions. As the cells enter this viable non-culturable state changes occur in:

- a) the ability of the cells to respire as shown by the changes in the cells electron transport system;
- b) proteins of the outer membrane particularly to OmpA which is important in maintaining the cell shape;
- c) the shape and size of the cells and;
- d) the activity of some enzymes involved in central metabolism such as the dehydrogenases but in those involved in nutrient scavenging such as alkaline phosphatase which must be part of the response of *E.coli* to starvation.

PAGINATION ERROR



Chapter X. References

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